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Attorney Docket **P0960R1D1C2**
PATENT

CERTIFICATION UNDER 37 CFR 1.10

EK517180319US: Express Mail Number **May 26, 2000: Date of Deposit**

I hereby certify that this Non-provisional Application Transmittal and the documents referred to as enclosed therein are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner of Patents, Washington, D.C. 20231.

Diane L. Marschang

Diane L. Marschang

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BOX PATENT APPLICATION
Assistant Commissioner of Patents
Washington, D.C. 20231

NON-PROVISIONAL APPLICATION TRANSMITTAL UNDER 37 CFR 1.53(b)

Transmitted herewith for filing is a non-provisional patent application:

Inventor(s) (or Application "Identifier"):

David V. Goeddel, Hillsborough, CA
Mike Rothe, San Mateo, CA

Title: **TRAF INHIBITORS**

1. Type of Application

[ ] This application is for an original, non-provisional application.

[ ] This is a non-provisional application claiming priority to provisional application no._ , filed _ the entire disclosure of which is hereby incorporated by reference.

[X] This is a [ ] continuation-in-part [X] continuation [ ] divisional application claiming priority to application Serial Number 09/459,952, filed December 13, 1999, which is a continuation application of Serial Number 09/020,467, filed February 9, 1998, which is a divisional application of Serial Number 08/700,749, filed August 14, 1996, claiming priority under Section 119(e) to provisional application Number 60/002,382, filed August 17, 1995, the entire disclosures of which are hereby incorporated by reference.

2. **Papers Enclosed Which Are Required For Filing Date Under 37 CFR 1.53(b) (Non-provisional)**

65 pages of specification
5 pages of claims
1 page(s) of abstract
5 sheet(s) of drawings
[] formal [X] informal

3. Declaration or Oath

(for new and CIP applications; also for Cont./Div. where inventor(s) are being added)
___ An executed declaration of the inventor(s) [] is enclosed [] will follow.

(for Cont./Div. where inventorship is the same or inventor(s) being deleted)
__X__ A copy of the executed declaration/oath filed in the prior application is enclosed (37 CFR 1.63(d)).

(for Cont./Div. where inventor(s) being deleted)
___ A signed statement is attached deleting inventor(s) named in the prior application (see 37 CFR 1.63(d)(2) and 1.33(b)).

4. Assignment

(for new and CIP applications)
___ An Assignment of the invention to GENENTECH, INC. [] is enclosed with attached Recordation Form Cover Sheet [] will follow.

(for cont./div.)
__X__ The prior application is assigned of record to Genentech, Inc. and Tularik, Inc.

5. Amendments (for continuation and divisional applications)

__X__ Cancel in this application original claims __1-34 and 38__ of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

__X__ A preliminary amendment is enclosed. (Claims added by this amendment have been properly numbered consecutively beginning with the number next following the highest numbered original claim in the prior application.)

Relate Back -- 35 U.S.C. 120 or 35 U.S.C. 119

__X__ Amend the specification by inserting before the first line the sentence:

--This is a

_____ non-provisional application
__X__ continuation
_____ divisional
_____ continuation-in-part

of co-pending application(s)

__X__ Serial Number 09/459,952, filed December 13, 1999, which is a continuation application of Serial Number 09/020,467, filed February 9, 1998, which is a divisional application of Serial Number 08/700,749, filed August 14, 1996, claiming priority under Section 119(e) to provisional application Number 60/002,382, filed August 17, 1995, which applications are incorporated herein by reference. --

_____ International Application _ filed on __ which designated the U.S., which application(s) is(are) incorporated herein by reference and to which application(s) priority is claimed under 35 USC §120.--

_____ provisional application No. _ filed __, the entire disclosure of which is hereby incorporated by reference and to which application(s) priority is claimed under 35 USC §119.--.

6. Fee Calculation (37 CFR 1.16)

The fee has been calculated as follows:

CLAIMS FOR FEE CALCULATION					
Number Filed		Number Extra		Rate	Basic Fee 37 CFR 1.16(a)
					$690.00
Total Claims	7	- 20 =	0	X $18.00	$0.00
Independent Claims	2	- 3 =	0	X $78.00	$0.00
__	Multiple dependent claim(s), if any			+ $260.00	$0.00
				Filing Fee Calculation	$690.00

7. Method of Payment of Fees

_X_ The Commissioner is hereby authorized to charge Deposit Account No. 07-0630 in the amount of $690.00. **A duplicate copy of this transmittal is enclosed.**

8. Authorization to Charge Additional Fees

_X_ The Commissioner is hereby authorized to charge any additional fees required under 37 CFR §1.16 and 1.17, or credit overpayment to Deposit Account No. 07-0630. **A duplicate copy of this sheet is enclosed.**

9. Additional Papers Enclosed

[] Information Disclosure Statement (37 CFR §1.98) w/ PTO-1449 and citations

[X] Submission of "Sequence Listing", Request to Use Computer Readable Copy from Applicants' prior Application, certificate re: sequence listing, and/or amendment pertaining thereto for biological invention containing nucleotide and/or amino acid sequence.

[] A new Power of Attorney or authorization of agent.

[] Other:

10. Maintenance of Copendency of Prior Application (for continuation and divisional applications)

*[This item **must** be completed and the necessary papers filed in the prior application if the period set in the prior application has run]*

_____ A petition, fee and/or response has been filed to extend the term in the pending prior application until

_____ A copy of the petition for extension of time in the ***prior*** application

is attached.

11. Correspondence Address:

X Address all future communications to:

GENENTECH, INC.
Attn: Diane L. Marschang
1 DNA Way
South San Francisco, CA 94080-4990
(650) 225-5416

Respectfully submitted,
GENENTECH, INC.

Date: May 26, 2000

By: Diane L. Marschang
Diane L. Marschang
Reg. No. 35,600

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Patent Docket P0960R1D1C2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Goeddel et al. Serial No.: To be assigned Filed: May 26, 2000 For: TRAF INHIBITORS	Group Art Unit: To be assigned Examiner: To be assigned

PRELIMINARY AMENDMENT

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

This Preliminary Amendment is being filed concurrently with Applicants' Rule 53(b) Continuation Application. Applicants respectfully request entry of the following preliminary amendment prior to examination on the merits.

In the Specification:

On page 3, lines 12-14, delete "U.S. application Serial No. 08/446,915 filed 22 May 1995 which is a continuation-in-part of application Serial No. 08/331,394 filed 28 October 1994 which, in turn, is a continuation-in-part of application Serial No. 08/250,858 filed 27 May 1994" and insert -- US Patent Nos. 5,670,319 issued September 23, 1997 and 5,708,142 issued January 13, 1998 --.

On page 7, please delete the text on line 9, and insert therefor - Figures 4A-C illustrate the inhibitory effect of I-TRAF on activation of NF-KB. Figure 4A shows that I-TRAF inhibits TRAF2-mediated NF-KB activation in a dose-dependent manner. Figures 4B and 4C show that NF-KB activation through both TNFR2 and CD40 is similarly blocked by increased expression of I-TRAF--.

On page 23, line 17, delete "homogenous" and insert -- homogeneous --.

On page 36, lines 17-18, delete "12301 Parklawn Drive, Rockville, MD" and insert - 10801 University Blvd., Manassas, Virginia --.

On page 40, line 4, delete "reported" and insert -- reporter --.

On page 10, line 10, delete "reported" and insert -- reporter --; on line 16, delete "4b" and insert -- 4C --; on line 17, delete "4c" and insert -- 4B --.

In the Claims:

Please cancel claims 1-34 and 38 without prejudice.

35. (Amended) A method of treating a tumor associated with Epstein-Barr virus comprising administering to a patient having developed or at risk of developing such tumor a therapeutically effective amount of an isolated I-TRAF polypeptide.

37. (Amended) A pharmaceutical formulation comprising a therapeutically effective amount of an isolated I-TRAF polypeptide in association with a pharmaceutically acceptable carrier.

Please add the following claims:

--39. The method of claim 35 wherein said I-TRAF polypeptide comprises the amino acid sequence of SEQ ID NO:5 or an allelic variant thereof.

40. The method of claim 35 wherein said I-TRAF polypeptide is encoded by a nucleic acid capable of hybridizing under stringent conditions to the complement of SEQ ID NO:6.

41. The method of claim 35 wherein said I-TRAF polypeptide is encoded by the nucleic acid of SEQ ID NO:6.

42. The method of claim 35 wherein radiation therapy, chemotherapy, or immunotoxin therapy is further administered to said patient. --

REMARKS

The amendments to the specification, as shown above, are directed to correcting formalities in the specification; specifically, bringing the specification into conformance with the numbering of the drawings, correcting certain inadvertent typographical errors, and indicating the current address of the ATCC depository. As such, it is believed that the amendments do not present substantive changes to the specification or introduce any new matter.

The amendments to claims 35 and 37, and added claims 39-42, are also fully supported by the specification as filed and do not introduce new matter.

Respectfully submitted,
GENENTECH, INC.

Date: May 26, 2000

By: Diane L Marschang
Diane L. Marschang
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PATENT DOCKET P0960R1D1

EXPRESS MAIL NO. TB 835 478 534 US

Date Mailed: 9 FEBRUARY 1998

TRAF INHIBITORS

This is a divisional of non-provisional application Serial No. 08/700,749 filed 14 August 1996, claiming priority under Section 119(e) to provisional Application Serial No. 60/002382 filed 17 August 1995, the entire disclosure of which is hereby incorporated by reference.

Field of the Invention

The present invention concerns the isolation, recombinant production and characterization of novel polypeptides which block signal transduction mediated by tumor necrosis factor associated factors (TRAFs), including TRAF2. In particular, this invention concerns novel proteins that act as specific inhibitors of TRAF2-dependent NF-κB activation signaled by certain members of the TNF receptor superfamily, such as TNF-R2 and CD40, methods and means for making them and their use in screening assays or as pharmaceuticals.

Background of the Invention

Tumor necrosis factor (TNF) is a cytokine produced mainly by activated macrophages which elicits a wide range of biological effects. These include an important role in endotoxic shock and in inflammatory, immunoregulatory, proliferative, cytotoxic, and anti-viral activities (reviewed by

Goeddel, D.V. *et al.*, Cold Spring Harbor Symposia on Quantitative Biology 51, 597-609 [1986]; Beutler, B. and Cerami, A., Ann. Rev. Biochem. 57, 505-518 [1988]; Old, L.J., Sci. Am. 258(5), 59-75 [1988]; Fiers, W. FEBS Lett. 285(2), 199-212 [1991]). The induction of the various cellular responses mediated by TNF is initiated by its interaction with two distinct cell surface receptors, an approximately 55 kDa receptor termed TNF-R1 and an approximately 75 kDa receptor termed TNF-R2. Human and mouse cDNAs corresponding to both receptor types have been isolated and characterized (Loetscher, H. *et al.*, Cell 61, 351 [1990]; Schall, T.J. *et al.*, Cell 61, 361 [1990]; Smith, C.A. *et al.*, Science 248, 1019 [1990]; Lewis, M. *et al.*, Proc. Natl. Acad. Sci. USA 88, 2830-2834 [1991]; Goodwin, R.G. *et al.*, Mol. Cell. Biol. 11, 3020-3026 [1991]. Both TNF-Rs share the typical structure of cell surface receptors including extracellular, transmembrane and intracellular regions. The extracellular portions of both receptors are found naturally also as soluble TNF-binding proteins (Nophar, Y. *et al.*, EMBO J. 9, 3269 [1990] and Kohno, T. *et al.*, Proc. Natl. Acad. Sci. U.S.A. 87, 8331 [1990]). The amino acid sequence of human TNF-R1 and the underlying nucleotide sequence are disclosed in EP 417,563 (published 20 March 1991), whereas EP 418,014 (published 20 March 1991) discloses the amino acid and nucleotide sequences of human TNF-R2.

Both TNF receptors are independently active in signaling TNF responses. Direct signaling by TNF-R2 has been observed in lymphoid cells in which TNF-R2 stimulates the proliferation of thymocytes and a murine cytotoxic T cell line CT6 (Tartaglia *et al.*, Proc. Natl. Acad. Sci. USA 88, 9292-9296 [1991]; Tartaglia *et al*, J. Immunol. 151, 4637-4641 [1993]). Both TNF-R1 and TNF-R2, along with other members of the TNF receptor superfamily, e.g. CD40, have been shown to independently mediate the activation of the transcription factor NF-κB (Lenardo & Baltimore, Cell 58: 227-229 [1989]; Lægreid, A., *et al.*, J. Biol. Chem. 269, 7785-7791 [1994]; Rothe *et al.*, Cell 78, 681-692 [1994]; Wiegmann *et al.*, J. Biol. Chem. 267, 17997-18001 [1992]). NF-κB is a member of the Rel family of transcriptional activators that control the expression of a variety of important cellular and viral genes (Lenardo & Baltimore, *supra*, and Thanos and Maniatis, Cell 80, 529-532 [1995]). TNF-R2 also mediates the transcriptional induction of the granulocyte-

macrophage colony stimulating factor (GM-CSF) gene (Miyatake *et al.*, EMBO J. 4: 2561-2568 [1985]; Stanley *et al.*, EMBO J. 4: 2569-2573 [1985]) and the A20 zinc finger protein gene (Opipari *et al.*, J. Biol. Chem. 265: 14705-14708 [1990]) in CT6 cells, and participates as an accessory component to TNF-R1 in the signaling of responses primarily mediated by TNF-R1, like cytotoxicity (Tartaglia, L.A. and Goeddel, D.V., Immunol. Today 13, 151-153 [1992]).

Recent research has lead to the isolation of polypeptide factors associated with the intracellular domain of the 75 kDa tumor necrosis factor receptor, TNF-R2 ("tumor necrosis factor receptor associated factors" or "TRAFs") which participate in the TNF-R2 signal transduction cascade. TRAF1 and TRAF2 were the first two identified members of this novel protein family containing a novel C-terminal homology region, the TRAF domain (Rothe *et al.*, Cell 78, 681-692 [1994]; U.S. application Serial No. 08/446,915 filed 22 May 1995 which is a continuation-in-part of application Serial No. 08/331,394 filed 28 October 1994 which, in turn, is a continuation-in-part of application Serial No. 08/250,858 filed 27 May 1994). A further TRAF domain protein, TRAF3 (originally termed CD40bp, CRAF, or LAP1) has also been identified (Hu *et al.*, J. Biol. Chem. 269, 30069 [1994]; Cheng *et al.*, Science 267, 1494 [1995], and Mosialos *et al.*, Cell 80, 389 [1995]). TRAFs transduce signals from TNF-R2, CD40 and presumably from other members of the TNF receptor superfamily that also includes the low affinity nerve growth factor receptor, the Fas antigen, CD27, CD30, OX40, 4-1BB, and TNFR-RP (Rothe *et al.*, *supra;* Hu *et al.*, J. Biol. Chem. 269, 30069-30072 [1994]; Cheng *et al.*, Science 267, 1494-1498 [1995]; Mosialos *et al.*, Cell 80, 389-399 [1995]; Rothe *et al.*, Science, *in press;* Smith *et al*, Cell 76, 959-962 [1994]). In addition to the shared conserved C-terminal TRAF domain that is involved in both receptor association and oligomerization, TRAF2 and TRAF3 each contain an N-terminal RING finger domain and five zinc finger structures of weak sequence similarity. CD40 and TNF-R2 interact directly with TRAF2 and indirectly with TRAF1 via a TRAF2/TRAF1 heterodimer. TRAF2 (or the TRAF2/TRAF1 heterodimer) is required for CD40- and TNF-R2-mediated activation of the transcription factor NF-κB. TRAF3 interacts with CD40 and self-associates, but does appear to not associate with TNF-R2,

TRAF1, or TRAF2. The role of TRAF3 in signal transduction is less well defined, but it may antagonize the effects of TRAF2 (Rothe *et al.*, Science, *in press*). The TRAF proteins also interact with the C-terminal cytoplasmic domain of the Epstein-Barr virus transforming protein LMP1 (Mosialos *et al.*, Cell 80, 389 [1995]). LMP1 is a dominant oncogene that has multiple downstream effects on cell growth and gene expression, at least some of which require NF-κB activation (Laherty *et al.*, J. Biol. Chem. 267, 24157 [1992]; Rowe *et al.*, J. Virol. 68, 5602 [1994]). TRAF2 is believed to be a common signal transducer for TNF-R2, CD40 and LMP1.

Summary of the Invention

The present invention is based on the identification, recombinant production and characterization of certain novel TRAF-interacting proteins named "inhibitors of TRAF" (I-TRAFs). More specifically, the present invention is based on the isolation of cDNAs encoding various forms of murine and human I-TRAFs encoding proteins with no significant sequence similarity to any known protein, and on the expression and characterization of the encoded I-TRAF proteins.

In one aspect, the invention concerns purified I-TRAF proteins which inhibit the interaction of TRAFs with members of the TNF receptor superfamily, such as TNF-R2, CD40, and/or other cellular proteins with which TRAF proteins are normally, directly or indirectly, associated, such as LMP1.

In particular, the invention concerns an isolated I-TRAF polypeptide comprising the amino acid sequence of a polypeptide selected from the group consisting of:

(a) a native sequence I-TRAF polypeptide,

(b) a polypeptide having at least about 70% amino acid sequence identity with the TRAF-binding domain of a native sequence I-TRAF polypeptide and capable of inhibiting a TRAF-mediated signaling event, and

(c) a fragment of a polypeptide of (a) or (b) capable of inhibiting a TRAF-mediated signaling event.

Preferably, the I-TRAF polypeptides of the present invention have an at least about 60% overall amino acid sequence identity with a native sequence I-TRAF polypeptide. The I-TRAF polypeptides preferably comprise a fragment of a native mammalian I-TRAF protein that is necessary and sufficient for association with a native TRAF protein. More preferably, the I-TRAF polypeptides comprise a fragment of a native mammalian I-TRAF or a polypeptide sufficiently homologous to retain the ability of interacting with the TRAF domain of a native mammalian TRAF protein. Even more preferably, the I-TRAF polypeptides comprise the N-terminal portion of a native mammalian I-TRAF capable of interaction with a native TRAF, preferably TRAF2. The mammalian proteins are preferably human.

In another aspect, the invention concerns an isolated nucleic acid molecule encoding I-TRAF proteins which inhibit the interaction of TRAFs with members of the TNF receptor superfamily, such as TNF-R2, CD40, and/or other intracellular proteins with which TRAF proteins are normally, directly or indirectly, associated, such as LMP1. The nucleic acid preferably comprises a nucleotide sequence encoding a 413 or 447 amino acid murine I-TRAF protein (SEQ. ID. NOs: 1 and 3) or a 425 amino acid human I-TRAF protein (SEQ. ID. NO: 5) or a functional derivative thereof, including the truncated murine protein shown in Fig. 1 (SEQ. ID. NO: 8). In another preferred embodiment, the nucleic acid is capable of hybridizing to the complement of any of the nucleic acid molecules represented by SEQ. ID. NOs: 2, 4 and 6.

The invention further concerns vectors, cells and organisms comprising such nucleic acid.

In a further aspect, the invention concerns a screening assay for identifying molecules that modulate the I-TRAF/TRAF binding. Preferably, the molecules either prevent I-TRAF/TRAF

interaction or prevent or inhibit dissociation of I-TRAF/TRAF complexes. The assay comprises the incubation of a mixture comprising TRAF and I-TRAF with a candidate molecule and detection of the ability of the candidate molecule to modulate I-TRAF/TRAF binding, e.g. to prevent the interaction of I-TRAF with TRAF or to prevent or inhibit the dissociation of I-TRAF/TRAF complexes. The screened molecules preferably are small molecule drug candidates.

In another aspect, the invention relates to an assay for identifying a molecule the signal transduction of which is mediated by the association of a TRAF with a cellular protein, comprising (a) incubating a mixture comprising a TRAF, and an I-TRAF with a candidate molecule, and (b) detecting the ability of the candidate molecule to release TRAF from the complex formed with I-TRAF a TRAF-mediated signaling event.

In yet another aspect, the invention concerns a method of treating a tumor associated with Epstein-Barr virus comprising administering to a patient having developed or at risk of developing such tumor a therapeutically effective amount of an I-TRAF. Pharmaceutical compositions comprising an I-TRAF as an active ingredient are also within the scope of the present invention.

The invention also concerns a method for modulating TRAF-mediated signal transduction in a cell comprising introducing into the cell a nucleic acid encoding an I-TRAF or a functional derivative.

Brief Description of Drawings

Figure 1 (a) shows the deduced amino acid sequences of murine and human I-TRAFs. The translation initiation codons of the murine I-TRAFα and -β splice variants are indicated by α and β, respectively. The splice junction is marked by an asterisk (*). All of the isolated human I-TRAF cDNA clones correspond to murine I-TRAFα. The initiator methionine of human I-TRAF is preceded by an upstream in-frame stop codon not present in the murine I-TRAFα cDNA clones.

The amino acid sequences of two additional splice variants of mouse I-TRAFα, ending in premature termination codons, are also listed.

Figure 1 (b) shows the results of Northern blot analysis of I-TRAF mRNA in multiple mouse tissues.

Figure 2 shows that I-TRAF associates with TRAF1, TRAF2 and TRAF3 in an *in vitro* binding assay.

Figure 3 shows the results of Western blot analysis which demonstrate that I-TRAF blocks the association of TRAF2 with TNF-R2.

Figure 4 illustrates the inhibitory effect of I-TRAF on activation of NF-κB.

SEQ. ID. NO: 1 shows the amino acid sequence of the 413 aa murine I-TRAF (murine I-TRAFα), starting with the amino acid marked "α" in Figure 1.

SEQ. ID. NO: 2 shows the nucleotide sequence of murine I-TRAFα. This sequence was obtained from a composite of several independent cloned sequenced.

SEQ. ID. NO: 3 shows the additional 34 amino acids present in the sequence of the 447 aa murine I-TRAF (murine I-TRAFβ), starting with the amino acid marked "β" in Figure 1.

SEQ. ID. NO: 4 shows the nucleotide sequence encoding the additional 34 amino acids of murine I-TRAFβ.

SEQ. ID. NO: 5 shows the amino acid sequence of full-length human I-TRAF.

SEQ. ID. NO: 6 shows the nucleotide sequence of full-length human I-TRAF.

SEQ. ID. NO: 7 is the complete, unedited nucleotide sequence of one full length clone encoding murine I-TRAFα (clone 8).

SEQ. ID. NO: 8 is the nucleotide sequence encoding the truncated murine I-TRAFα protein ending by the sequence VTVLH shown in Figure 1 (clone 23).

Detailed Description of the Invention

A. Definitions

The phrases "inhibitor of TRAF", "inhibitor of TRAF protein", "inhibitor of TRAF polypeptide", "I-TRAF protein", "I-TRAF polypeptide" and "I-TRAF" are used interchangeably and refer to a native sequence polypeptide which inhibits the interaction of a native TRAF with a cell surface receptor that triggers TRAF-mediated NF-κB activation cascades, such as a member of the TNF receptor superfamily (e.g. TNF-R2, CD40 and/or CD30) and/or with another cellular protein with which the native TRAF is normally (directly or indirectly) associated, provided that such I-TRAF polypeptide is other than a blocking antibody, and functional derivatives thereof. The I-TRAF protein preferably exerts its inhibitory function by binding to a TRAF protein, such as TRAF1, TRAF2 and/or TRAF3, keeping it in a latent state from which the TRAF protein can be released, for example, by ligand-induced receptor aggregation. The phrases "native sequence polypeptide" and "native polypeptide" and their grammatical variants are used interchangeably and designate a polypeptide as occurring in nature in any cell type of any human or non-human animal species, with or without the initiating methionine, whether purified from native source, synthesized, produced by recombinant DNA technology or by any combination of these and/or other methods. Native sequence I-TRAF polypeptides specifically include the 413 amino acid murine I-TRAF (I-TRAFα; SEQ. ID. NO: 1), the 447 amino acid murine I-TRAF (I-TRAFβ; SEQ. ID. NO: 3), and the 425 amino acid human I-TRAF (SEQ. ID. NO: 5), and their additional, naturally occurring alternative splice and allelic variants. Native I-TRAF polypeptides from other mammalian species, such as porcine, canine, equine, etc. are also included.

The phrases "TRAF", "TRAF protein" and "TRAF polypeptide" are used interchangeably and refer to a native sequence factor capable of specific association with the intracellular domain of a native member of the TNF receptor superfamily (such as TNF-R2, CD40 or CD30), and functional derivatives of such native factor. In the context of this definition, the phrase "specific association" is used in the broadest sense, and includes direct binding to a site or region within the intracellular

domain of a native TNF receptor superfamily member of the human or of any other animal species, and indirect association mediated by a further molecules, such as another TRAF. The phrase "native sequence factor" is defined in an analogous manner to the definition of "native sequence polypeptide" provided above. The native sequence TRAF polypeptides specifically include the native murine TRAF1 and TRAF2 polypeptides disclosed in Rothe *et al.*, Cell 78, 681-692 [1994]), TRAF3 as disclosed in Hu *et al.*, *supra*; Cheng *et al.*, *supra*; and Mosialos *et al.*, *supra*, and their equivalents in human and other animal species.

A "functional derivative" of a native polypeptide is a compound having a qualitative biological activity in common with the native polypeptide. For the purpose of the present invention, a "functional derivative" of a native I-TRAF is defined by its ability to inhibit the association of a TRAF with a member of the TNF receptor superfamily with which the TRAF is naturally associated, with the *proviso* that such functional derivatives are not (anti-TRAF or anti-TNF receptor superfamily member) blocking antibodies. Preferably, a functional derivative binds to a native TRAF polypeptide, such as TRAF1, TRAF2 and/or TRAF3 thereby (reversibly or irreversibly) preventing its interaction with cell surface receptors (e.g., TNF-R2, CD40, CD30) that trigger TRAF-mediated NF-κB activation cascades. In a particularly preferred embodiment, the prevention of TRAF-mediated signaling is reversible, and the TRAFs can be released from their latent, inhibited state, e.g. by ligand-mediated receptor aggregation. The functional derivatives preferably have at least about 60%, more preferably at least about 70%, even more preferably at least about 80%, most preferably at least about 90% overall amino acid sequence identity with a native sequence I-TRAF polypeptide, preferably a human I-TRAF. Even more preferably, the functional derivatives show at least about 70%, more preferably at least about 80% and most preferably at least about 90% amino acid sequence identity with the TRAF-binding domain of a native sequence I-TRAF polypeptide. Fragments of native sequence I-TRAF polypeptides from various mammalian species and sequences homologous to such fragments constitute another preferred group of I-TRAF functional derivatives. Such fragments preferably include the N-terminal region of a native sequence I-TRAF which is

necessary and sufficient for TRAF binding, such as amino acids 35-236 of the mouse I-TRAF sequences represented in SEQ. ID. NO: 1 or 3, and the equivalent part of a human I-TRAF sequence, or show at least about 70%, more preferably at least about 80%, most preferably at least about 90% sequence identity with the TRAF-binding N-terminal portion of a native sequence I-TRAF. Another preferred group of I-TRAF functional derivatives is encoded by nucleic acid hybridizing under stringent conditions to the complement of nucleic acid encoding a native I-TRAF polypeptide.

Functional derivatives of native TRAF polypeptides, as defined for the purpose of the present invention, are characterized by retaining the ability of native TRAFs to associate (directly or indirectly) with the intracellular domain of a member of the TNF receptor superfamily, such as TNF-R2, CD40 and/or CD30. Such TRAF functional derivatives preferably retain or mimic the region(s) within a native TRAF sequence that directly participate(s) in association with the intracellular domain of a TNF receptor superfamily member and/or in homo- or heterodimerization, along with the region(s) to which a native I-TRAF polypeptide binds. A preferred group of TRAF functional derivatives shows at least about 60%, more preferably at least about 70%, even more preferably at least about 80%, most preferably at least about 90% overall sequence identity with a native sequence TRAF. Other preferred functional derivatives are or comprise the conserved TRAF domains of native sequence TRAF proteins. Within this group, TRAF fragments comprising at least amino acids 264 to 501 of the native TRAF2 amino acid sequence are particularly preferred. Also preferred are TRAF variants which show at least about 70%, more preferably at least about 80%, most preferably at least about 80% amino acid sequence identity with the TRAF region of a native sequence TRAF protein, preferably TRAF2. A further preferred group of TRAF functional derivatives is encoded by nucleic acid capable of hybridizing under stringent conditions to the complement of a native sequence TRAF polypeptide of any mammalian species, including humans.

"Identity" or "homology" with respect to a native polypeptide or polypeptide and its functional derivative herein is the percentage of amino acid residues in the candidate sequence that are identical with the residues of a corresponding native polypeptide, after aligning the sequences and introducing gaps, if necessary, to achieved the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. Methods and computer programs for the alignment are well known in the art,

"Stringent conditions" can be provided in a variety of ways, such as by overnight incubation at 42°C in a solution comprising: 20% formamide, 5xSSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured, sheared salmon sperm DNA. Alternatively, the stringent conditions are characterized by a hybridization buffer comprising 30% formamide in 5x SSPE (0.18 M NaCl, 0.01 M $NaPO_4$, pH 7.7, 0.0001 M EDTA) buffer at a temperature of 42°C, and subsequent washing at 42°C with 0.2x SSPE. Preferably, stringent conditions involve the use of a hybridization buffer comprising 50% formamide in 5 x SSPE at a temperature of 42°C and washing at the same temperature with 0.2 x SSPE.

An "isolated" polypeptide or nucleic acid is unaccompanied with at least some of the material with which it is associated in its native environment. An isolated polypeptide constitutes at least about 2% by weight, and preferably at least about 5% by weight of the total protein in a given sample. An isolated nucleic acid constitutes at least about 0.5 % by weight, and preferably at least about 5% by weight of the total nucleic acid present in a given sample.

The term "antibody" is used in the broadest sense and specifically covers single monoclonal antibodies (including agonist and antagonist antibodies), antibody compositions with polyepitopic specificity, as well as antibody fragments (e.g., Fab, $F(ab')_2$, and Fv), so long as they exhibit the desired biological activity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler & Milstein, Nature 256:495 (1975), or may be made by recombinant DNA methods [see, e.g. U.S. Patent No. 4,816,567 (Cabilly *et al.*)].

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity [U.S. Patent No. 4,816,567; Cabilly *et al.*; Morrison *et al.*, Proc. Natl. Acad. Sci. USA 81, 6851-6855 (1984)].

"Humanized" forms of non-human (e.g. murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding

subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details see: Jones *et al.*, Nature 321, 522-525 (1986); Reichmann *et al.*, Nature 332, 323-329 (1988); Presta, Curr. Op. Struct. Biol. 2 593-596 (1992) and U.S. Patent No. 5,225,539 (Winter) issued July 6, 1993.

A "vector" as defined for the purpose of the present invention refers to a DNA construct containing a DNA sequence which is operably linked to a suitable control sequence capable of effecting the expression of the DNA in a suitable host. Such control sequences include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribosome binding sites, and sequences which control the termination of transcription and translation. The vector may be a plasmid, a phage particle, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, in some instances, integrate into the genome itself. In the present specification "plasmid" and "vector" are sometimes used interchangeably as the plasmid is the most commonly used form of vector at present. However, the invention is intended to include

such other forms of vectors which serve equivalent functions and which are, or become, known in the art. Preferred expression vectors for mammalian cell culture expression are based on pRK5 (EP 307,247; Rothe *et al.*, Cell, *supra*) and pSVI6B (PCT Publication No WO 91/08291).

In the context of the present invention the expressions "cell", "cell line", and "cell culture" are used interchangeably, and all such designations include progeny. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological property, as screened for in the originally transformed cell, are included.

The "host cells" used in the present invention generally are prokaryotic or eukaryotic hosts. Such host cells are, for example, disclosed in U.S. Patent No. 5,108,901 issued 28 April 1992, and in copending application Serial No. 08/446,915 filed 22 May 1995 and its parent applications. Suitable prokaryotes include gram negative or gram positive organisms, for example *E. coli* or bacilli. A preferred cloning host is *E. coli 294* (ATCC 31,446) although other gram negative or gram positive prokaryotes such as *E. coli* B, *E. coli* x 1776 (ATCC 31,537), *E. coli* W3110 (ATCC 27,325), *Pseudomonas* species, or *Serratia Marcesans* are suitable. In addition to prokaryotes, eukaryotic microbes such as filamentous fungi and yeasts are suitable hosts for appropriate vectors of the invention. *Saccharomyces cerevisiae,* or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species and strains are commonly available and useful herein, such as those disclosed in the above-cited patent and patent applications. A preferred yeast strain for the present invention is *Saccharomyces cerevisiae* HF7c (CLONTECH).

Suitable host cells may also derive from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture, although cells from mammals such as

humans are preferred. Examples of invertebrate cells include plant and insect cells, see, e.g. Luckow *et al.*, Bio/Technology 6, 47-55 (1988); Miller *et al.* in: Genetic Engineering, Setlow *et al.*, eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda *et al.*, Nature 315, 592-594 (1985). Interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) is *per se* known. See, Tissue Culture, Academic Press, Kruse and Patterson, eds. (1973). Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney cell line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al.*, J. Gen. Virol. 36, 59 [1977]); baby hamster kidney cells 9BHK, (ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA 77, 4216 [1980]); mouse sertolli cells (TM4, Mather, Biol. Reprod. 23, 243-251 [1980]); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*, Annals N.Y. Acad. Sci. 383, 44068 [1982]); MRC 5 cells; FS4 cells; and a human hepatoma cell line (Hep G2). Preferred host cells are human embryonic kidney 293 and Chinese hamster ovary cells. Particularly preferred for the present invention are the murine interleukin-2-dependentcytotoxic T cell line CT6, and the human embryonic kidney cell line 293, which are maintained as described in Tartaglia *et al.*, Proc. Natl. Acad. Sci. USA 88, 9292-9296 (1991) and Pennica *et al.*, J. Biol. Chem. 267, 21172-21178 (1992).

"Dissociation" is the process by which two molecules cease to interact: the process occurs at a fixed average rate under specific physical conditions.

B. Identification and purification of native I-TRAFs

The native I-TRAF polypeptides may, for example, be identified and purified from certain tissues which possess a TRAF (e.g. TRAF2) mRNA and to express it at a detectable level. Murine

I-TRAF can, for example, be obtained from the murine fetal liver stromal cell line 7-4 (FL; Rothe *et al.*, Cell *supra*) or from murine peripheral lymph nodes (PLN; U.S. Patent No. 5,304,640 issued April 19, 1994). Human I-TRAF can, for example, be purified from human peripheral lymph nodes. I-TRAF mRNA is also present and expressed in brain, spleen, lung, liver, skeletal muscle, kidney and testis tissues (see Figure 1b), along with TRAF, e.g. TRAF2 mRNAs. Native I-TRAFs can, for example, be identified and purified from tissues expressing their mRNAs based upon their ability to bind TRAF2 (or another TRAF, e.g. TRAF1). Native I-TRAFs will coprecipitated with immunoprecipitated TRAF2. In a preferred embodiment, radiolabeled TRAF2 or a derivative is immunoprecipitated with protein A-agarose (Oncogene Science) or with protein A-Sepharose (Pharmacia). The immunoprecipitate is then analyzed by autoradiography or fluorography, depending on the radiolabel used. The I-TRAF proteins will coprecipitate with the TRAF2 or its derivative, and can be further purified by methods known in the art, such as purification on an affinity column. For large-scale purification a scheme similar to that described by Smith and Johnson, Gene 67, 31-40 (1988) can be used. A cell lysate containing the I-TRAF(s) to be purified is applied to a glutathione-S-transferase (GST)-TRAF fusion protein affinity column. Protein(s) bound to the column is/are eluted, precipitated and isolated by SDS-PAGE under reducing conditions, and visualized, e.g. by silver staining. GST gene fusion vectors (pGEX vectors) as well as kits for cloning and expression of GST fusion systems are commercially available from Pharmacia (see Pharmacia Catalog, 1994, pages 133; and 142-143).

C. Recombinant production of I-TRAF polypeptides

Preferably, the I-TRAF polypeptides are prepared by standard recombinant procedures by culturing cells transfected to express I-TRAF polypeptide nucleic acid (typically by transforming the cells with an expression vector) and recovering the polypeptide from the cells. However, it is envisioned that the I-TRAF polypeptides may be produced by homologous recombination, or by recombinant production methods utilizing control elements introduced into cells already containing DNA encoding an TRAF polypeptide. For example, a powerful promoter/enhancer element, a

suppressor, or an exogenous transcription modulatory element may be inserted in the genome of the intended host cell in proximity and orientation sufficient to influence the transcription of DNA encoding the desired I-TRAF polypeptide. The control element does not encode the I-TRAF polypeptide, rather the DNA is indigenous to the host cell genome. One next screens for cells making the polypeptide of this invention, or for increased or decreased levels of expression, as desired. General techniques of recombinant DNA technology are, for example, disclosed in Sambrook *et al.*, Molecular Cloning: A laboratory Manual, Second Edition (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press) 1989.

1. Isolation of DNA encoding the I-TRAF polypeptides

For the purpose of the present invention, DNA encoding a I-TRAF polypeptide may, for example, be obtained from cDNA libraries prepared from tissue believed to possess a TRAF mRNA and to express it at a detectable level. For example, cDNA library can be constructed by obtaining polyadenylated mRNA from a cell line known to express a TRAF protein, and using the mRNA as a template to synthesize double stranded cDNA. Human and non-human cell lines and tissues suitable for this purpose have been listed hereinabove. Alternatively, DNA encoding new I-TRAF polypeptides can be obtained from cDNA libraries prepared from tissue known to express a previously identified I-TRAF polypeptide at a detectable level. The I-TRAF polypeptide genes can also be obtained from a genomic library, such as a human genomic cosmid library.

Libraries, either cDNA or genomic, are screened with probes designed to identify the gene of interest or the protein encoded by it. For cDNA expression libraries, suitable probes include monoclonal and polyclonal antibodies that recognize and specifically bind to an I-TRAF polypeptide. For cDNA libraries, suitable probes include carefully selected oligonucleotide probes (usually of about 20-80 bases in length) that encode known or suspected portions of an I-TRAF polypeptide from the same or different species, and/or complementary or homologous cDNAs or fragments thereof that encode the same or a similar gene. Appropriate probes for screening genomic

DNA libraries include, without limitation, oligonucleotides, cDNAs, or fragments thereof that encode the same or a similar gene, and/or homologous genomic DNAs or fragments thereof. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures as described in Chapters 10-12 of Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, New York, Cold Spring Harbor Laboratory Press, (1989).

A preferred method of practicing this invention is to use carefully selected oligonucleotide sequences to screen cDNA libraries from various tissues. The oligonucleotide sequences selected as probes should be sufficient in length and sufficiently unambiguous that false positives are minimized. The actual nucleotide sequence(s) is/are usually designed based on regions of an I-TRAF which have the least codon redundance. The oligonucleotides may be degenerate at one or more positions. The use of degenerate oligonucleotides is of particular importance where a library is screened from a species in which preferential codon usage is not known.

The oligonucleotide must be labeled such that it can be detected upon hybridization to DNA in the library being screened. The preferred method of labeling is to use ATP (e.g., $\gamma^{32}P$) and polynucleotide kinase to radiolabel the 5' end of the oligonucleotide. However, other methods may be used to label the oligonucleotide, including, but not limited to, biotinylation or enzyme labeling.

cDNAs encoding I-TRAFs can also be identified and isolated by other known techniques of recombinant DNA technology, such as by direct expression cloning or by using the polymerase chain reaction (PCR) as described in U.S. Patent No. 4,683,195, issued 28 July 1987, in section 14 of Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, second edition, Cold Spring Harbor Laboratory Press. New York, 1989, or in Chapter 15 of Current Protocols in Molecular Biology, Ausubel *et al.* eds., Greene Publishing Associates and Wiley-Interscience 1991. This method requires the use of oligonucleotide probes that will hybridize to DNA encoding an I-TRAF.

According to a preferred method for practicing the invention, the coding sequences for I-TRAF proteins can be identified in a recombinant cDNA library or a genomic DNA library based upon their ability to interact with TRAF proteins, e.g. TRAF2. For this purpose one can use the yeast genetic system described by Fields and co-workers [Fields and Song, Nature (London) 340, 245-246 (1989); Chien *et al.*, Proc. Natl. Acad. Sci. USA 88, 9578-9582 (1991)] as disclosed by Chevray and Nathans [Proc. Natl. Acad. Sci. USA 89, 5789-5793 (1992)]. Many transcriptional activators, such as yeast GAL4, consist of two physically discrete modular domains, one acting as the DNA-binding domain, while the other one functioning as the transcription activation domain. The yeast expression system described in the foregoing publications (generally referred to as the "two-hybrid system") takes advantage of this property, and employs two hybrid proteins, one in which the target protein is fused to the DNA-binding domain of GAL4, and another, in which candidate activating proteins are fused to the activation domain. The expression of a GAL1-*lacZ* reporter gene under control of a GAL4-activated promoter depends on reconstitution of GAL4 activity via protein-protein interaction. Colonies containing interacting polypeptides are detected with a chromogenic substrate for β-galactosidase. A complete kit (MATCHMAKER™) for identifying protein-proteininteractions between two specific proteins using the two-hybrid technique is commercially available from Clontech. This system can also be extended to map protein domains involved in specific protein interactions as well as to pinpoint amino acid residues that are crucial for these interactions. Yeast two-hybrid cloning can be performed as described in Rothe *et al.*, *supra*, using TRAF2 or its TRAF domain in the GAL4 DNA-binding domain vector pPC97 (Chevray and Nathans, *supra*) as bait.

Once the sequence is known, the gene encoding a particular I-TRAF polypeptide can also be obtained by chemical synthesis, following one of the methods described in Engels and Uhlmann, Angew. Chem. Int. Ed. Engl. 28, 716 (1989). These methods include triester, phosphite, phosphoramidite and H-phosphonate methods, PCR and other autoprimer methods, and oligonucleotide syntheses on solid supports.

2. Amino acid sequence variants of native I-TRAF proteins or fragments

Amino acid sequence variants of native I-TRAFs and I-TRAF fragments are prepared by methods known in the art by introducing appropriate nucleotide changes into a native or variant I-TRAF DNA, or by *in vitro* synthesis of the desired polypeptide. There are two principle variables in the constructions of amino acid sequence variants: the location of the mutation site and the nature of the mutation. With the exception of naturally-occurring alleles, which do not require the manipulation of the DNA sequence encoding the I-TRAF, the amino acid sequence variants of I-TRAF polypeptides are preferably constructed by mutating the DNA, either to arrive at an allele or an amino acid sequence variant that does not occur in nature. Methods for identifying target residues within native proteins and for making amino acid sequence variants are well known in the art, and are, for example, disclosed in U.S. Patent No. 5,108,901 issued 28 April 1992, and in copending application Serial No. 08/446,915 filed 22 May 1995 and its parent applications. The preferred techniques include alanine-scanning mutagenesis, PCR mutagenesis, cassette mutagenesis, the phageamid display method, details of which are also found in general textbooks, such as, for example, Sambrook *et al.*, *supra*, and Current Protocols in Molecular Biology, Ausubel *et al.*, eds., *supra.*

A preferred group of the I-TRAF amino acid sequence variants of the present invention comprises the substitution, insertion and/or deletion of one or more amino acids in a region directly involved in TRAF-binding. Amino acid alterations within this region are expected to result in genuine changes in the I-TRAF/TRAF binding affinity and may yield variants with stronger or weaker binding affinity than native I-TRAFs, as desired.

Naturally-occurring amino acids are divided into groups based on common side chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;

(2) neutral hydrophobic: cys, ser, thr;

(3) acidic: asp, glu;

(4) basic: asn, gln, his, lys, arg;

(5) residues that influence chain orientation: gly, pro; and

(6) aromatic: trp, tyr, phe.

Conservative substitutions involve exchanging a member within one group for another member within the same group, whereas non-conservative substitutions will entail exchanging a member of one of these classes for another. Variants obtained by non-conservative substitutions are expected to result in more significant changes in the biological properties/function of the obtained variant.

Amino acid sequence deletions generally range from about 1 to 30 residues, more preferably about 1 to 10 residues, and typically are contiguous. Deletions are preferably introduced into regions not directly involved in the interaction with TRAF, although deletions, optionally in combination with other types of mutations, in the TRAF-binding domain are also contemplated.

Amino acid insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions (i.e. insertions within the I-TRAF protein amino acid sequence) may range generally from about 1 to 10 residues, more preferably 1 to 5 residues, more preferably 1 to 3 residues. Examples of terminal insertions include the I-TRAF polypeptides with an N-terminal methionyl residue, an artifact of its direct expression in bacterial recombinant cell culture, and fusion of a heterologous N-terminal signal sequence to the N-terminus of the I-TRAF molecule to facilitate the secretion of the mature I-TRAF from recombinant host cells. Such signal sequences will generally be obtained from, and thus homologous to, the intended host cell species. Suitable sequences include STII or Ipp for E. coli, alpha factor for yeast, and viral signals such as herpes gD for mammalian cells.

Other insertional variants of the native I-TRAF molecules include the fusion of the N- or C-terminus of the I-TRAF molecule to immunogenic polypeptides, e.g. bacterial polypeptides such as beta-lactamase or an enzyme encoded by the E. coli trp locus, or yeast protein, and C-terminal fusions with proteins having a long half-life such as immunoglobulin regions (preferably immunoglobulin constant regions to yield immunoadhesins), albumin, or ferritin, as described in WO 89/02922 published on 6 April 1989. For the production of immunoglobulin fusions see also US Patent No. 5,428,130 issued June 27, 1995.

Since it is often difficult to predict in advance the characteristics of a variant TRAF, it will be appreciated that screening will be needed to select the optimum variant. For this purpose biochemical or other screening assays, such as those described hereinbelow, will be readily available.

Preferred amino acid sequence variants of native sequence I-TRAF polypeptides have the sequences not required for TRAF-binding (e.g. most of amino acids from position 237 to the C-terminus of the murine I-TRAF sequence) deleted, leaving the TRAF-interacting region as the sole functionally intact domain. Such variants may additionally have amino acid substitutions, insertions and/or deletions within the TRAF-interacting region, in order to optimize their binding properties, stability or other characteristics.

3. Insertion of DNA into a cloning/expression vehicle

Once the nucleic acid of a native or variant I-TRAF is available, it is generally ligated into a replicable expression vector for further cloning (amplification of the DNA) or expression.

Expression and cloning vectors are well known in the art and contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. The selection of an appropriate vector will depend on 1) whether it is to be used for DNA amplification or for DNA expression, 2) the size of the DNA to be inserted into the vector, and 3) the host cell to be transformed with the

vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the host cell for which it is compatible. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Suitable expression vectors, for use in combination with a variety of host cells, are well known in the art, and their preferred representatives have been listed hereinabove.

The host cells are then transformed, cultured, and the gene amplification/expression is detected by methods well known in the art, such as those disclosed in Sambrook *et al.*, *supra*, and Ausubel *et al.*, *supra*.

4. Isolation and purification of the I-TRAF polypeptides

The I-TRAF polypeptides are typically recovered from lysates of recombinant host cells. When I-TRAF is expressed in a recombinant host cell other than one of human origin, it is completely free of proteins or polypeptides of human origin. However, it is necessary to purify the I-TRAF protein from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogenous to the I-TRAF. As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. The membrane and soluble fractions are then separated. The I-TRAF protein may then be purified from the soluble protein fraction. The following procedures are exemplary of suitable purification procedures: fractionation or immunoaffinity on ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminant such as IgG. The preferred scheme for purifying native I-TRAFs from cells in which they naturally occur is equally suitable for the purification of recombinant I-TRAFs, including functional derivatives of the native molecules, from recombinant host cells.

D. Covalent modifications of I-TRAF polypeptides

Covalent modifications of I-TRAF are included within the scope herein. Such modifications are traditionally introduced by reacting targeted amino acid residues of the I-TRAF with an organic derivatizing agent that is capable of reacting with selected sides or terminal residues, or by harnessing mechanisms of post-translational modifications that function in selected recombinant host cells. The resultant covalent derivatives are useful in programs directed at identifying residues important for biological activity, for immunoassays of the I-TRAF, or for the preparation of anti-I-TRAF antibodies for immunoaffinity purification. For example, complete inactivation of the biological activity of the protein after reaction with ninhydrin would suggest that at least one arginyl or lysyl residue is critical for its activity, whereafter the individual residues which were modified under the conditions selected are identified by isolation of a peptide fragment containing the modified amino acid residue. Such modifications are within the ordinary skill in the art and are performed without undue experimentation.

Cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α-bromo-β-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl

residues. Other suitable reagents for derivatizing α-amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4-pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R'-N=C=N-R') such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl, threonyl or tyrosyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 [1983]), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group. The molecules may further be covalently linked to nonproteinaceous polymers, e.g. polyethylene glycol, polypropylene glycol or polyoxyalkylenes, in the manner set forth in U.S.S.N. 07/275,296 or U.S. patents 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

Derivatization with bifunctional agents is useful for preparing intramolecular aggregates of I-TRAFs with polypeptides as well as for cross-linking the I-TRAF polypeptide to a water insoluble support matrix or surface for use in assays or affinity purification. In addition, a study of interchain cross-links will provide direct information on conformational structure. Commonly used cross-linking agents include 1,1-bis(diazoacetyl)-2-phenylethane glutaraldehyde, N-hydroxysuccinimide esters, homobifunctional imidoesters, and bifunctional maleimides. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable intermediates which are capable of forming cross-links in the presence of light. Alternatively, reactive water insoluble matrices such as cyanogen bromide activated carbohydrates and the systems reactive substrates described in U.S. Patent Nos. 3,959,642; 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; 4,055,635; and 4,330,440 are employed for protein immobilization and cross-linking.

Certain post-translational modifications are the result of the action of recombinant host cells on the expressed polypeptide. Glutaminyl and aspariginyl residues are frequently post-translationally deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl, threonyl or tyrosyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)].

The TRAF may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, in colloidal drug delivery systems (e.g. liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, 16th Edition, Osol, A., Ed. (1980).

E. Use of I-TRAF polypeptides and nucleic acid encoding them

The nucleic acids encoding the I-TRAF polypeptides of the present invention find a variety of applications on their own, including use as translatable transcripts, hybridization probes for identifying nucleic acid encoding I-TRAFs from other species and/or structurally related polypeptides, PCR primers, and therapeutic nucleic acids, including gene therapy applications. In gene therapy applications, genes are introduced into cells in order to achieve *in vivo* synthesis of a therapeutically effective genetic product, for example for replacement of a defective gene. "Gene therapy" includes both conventional gene therapy where a lasting effect is achieved by a single treatment, and the administration of gene therapeutic agents, which involves the one time or repeated administration of a therapeutically effective DNA or mRNA. Antisense RNAs and DNAs can be used as therapeutic agents for blocking the expression of certain genes *in vivo*. It has already been shown that short antisense oligonucleotides can be imported into cells where they act as inhibitors, despite their low intracellular concentrations caused by their restricted uptake by the cell membrane. (Zamecnik *et al.*, Proc. Natl. Acad. Sci. USA 83, 4143-4146 [1986]). The oligonucleotides can be

modified to enhance their uptake, e.g. by substituting their negatively charged phosphodiester groups by uncharged groups.

There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells *in vitro*, or *in vivo* in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells *in vitro* include the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran, the calcium phosphate precipitation method, etc. The currently preferred *in vivo* gene transfer techniques include transfection with viral (typically retroviral) vectors and viral coat protein-liposome mediated transfection (Dzau *et al.*, Trends in Biotechnology 11, 205-210 [1993]). In some situations it is desirable to provide the nucleic acid source with an agent that targets the target cells, such as an antibody specific for a cell surface membrane protein or the target cell, a ligand for a receptor on the target cell, etc. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake, e.g. capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, proteins that target intracellular localization and enhance intracellular half-life. The technique of receptor-mediated endocytosis is described, for example, by Wu *et al.*, J. Biol. Chem. 262, 4429-4432 (1987); and Wagner *et al.*, Proc. Natl. Acad. Sci. USA 87, 3410-3414 (1990). For review of the currently known gene marking and gene therapy protocols see Anderson *et al.*, Science 256, 808-813 (1992).

The I-TRAF polypeptides are useful in assays for identifying lead compounds for therapeutically active agents that modulate I-TRAF/TRAF binding. Specifically, lead compounds that either prevent the formation of I-TRAF/TRAF complexes or prevent or inhibit dissociation of the I-TRAF/TRAF complexes already formed can be conveniently identified. Molecules preventing the interaction of I-TRAF and TRAF may find utility under circumstances when boosting of the immune system is desirable. Inhibitors of the dissociation of I-TRAF/TRAF complexes may be

useful as immunosuppressants or antiinflammatory agents. Screening assays can also be designed to find lead compounds that mimic the biological activity of a native ligand of a TNF receptor superfamily member with which a TRAF protein is associated, e.g. TNF, CD40 ligand, CD30 ligand, etc. These screening methods involve assaying the candidate agents for their ability to release TRAFs from I-TRAF inhibition.

The screening assays of the present invention are amenable to high-throughput screening of chemical libraries, and are particularly suitable for identifying small molecule drug candidates. Small molecules, which are usually less than 10 K molecular weight, are desirable as therapeutics since they are more likely to be permeable to cells, are less susceptible to degradation by various cellular mechanisms, and are not as apt to elicit an immune response as proteins. Small molecules include but are not limited to synthetic organic or inorganic compounds. Many pharmaceutical companies have extensive libraries of such molecules, which can be conveniently screened by using the assays of the present invention.

The assays can be performed in a variety of formats, including protein-protein binding assays, biochemical screening assays, immunoassays, cell based assays, etc. Such assay formats are well known in the art.

The assay mixture typically contains an I-TRAF and a TRAF protein with which I-TRAF is normally associated (e.g. TRAF2 or TRAF3), usually in an isolated, partially pure or pure form. One or both of these components may be fused to another peptide or polypeptide, which may, for example, provide or enhance protein-protein binding, improve stability under assay conditions, etc. In addition, one of the components usually comprises or is coupled to a detectable label. The label may provide for direct detection by measuring radioactivity, luminescence, optical or electron density, etc., or indirect detection such as an epitope tag, an enzyme, etc. The assay mixture additionally comprises a candidate pharmacological agent, and optionally a variety of other

components, such as salts, buffers, carrier proteins, e.g. albumin, detergents, protease inhibitors, nuclease inhibitors, antimicrobial agents, etc., which facilitate binding, increase stability, reduce non-specific or background interactions, or otherwise improve the efficiency or sensitivity of the assay.

To screen for inhibitors of I-TRAF/TRAF binding, the assay mixture is incubated under conditions whereby, but for the presence of the candidate pharmacological agent, the I-TRAF specifically binds the TRAF protein with a reference binding affinity. The mixture components can be added in any order that provides for the requisite binding. Incubation may be performed at any temperature which facilitates optimal binding, typically between about 4° and 40°C, more commonly between about 15° and 40°C. Incubation periods are likewise selected for optimal binding but also minimized to facilitate rapid, high-throughput screening, and are typically between about 0.1 and 10 hours, preferably less than 5 hours, more preferably less than 2 hours. After incubation, the effect of the candidate pharmacological agent on the I-TRAF/TRAF binding is determined in any convenient way. For cell-free binding-type assays, a separation step is often used to separate bound and unbound components. Separation may, for example, be effected by precipitation (e.g. TCA precipitation, immunoprecipitation, etc.), immobilization (e.g. on a solid substrate), followed by washing. The bound protein is conveniently detected by taking advantage of a detectable label attached to it, e.g. by measuring radioactive emission, optical or electron density, or by indirect detection using, e.g. antibody conjugates.

Compounds which inhibit or prevent the dissociation of the I-TRAF/TRAF complexes can be conveniently identified by forming the I-TRAF/TRAF complex in the absence of the candidate pharmacological agent, adding the agent to the mixture, and changing the conditions such that, but for the presence of the candidate agent, TRAF would be released from the complex. This can, for example, be achieved by changing the incubation temperature or by adding to the mixture a compound which, in the absence of the candidate pharmacological agent, would release TRAF from

its complexed form. As an example, this compound can be a native ligand the signal transduction of which is mediated by TRAF, e.g. TNF, a CD40 ligand, a CD30 ligand, etc. The concentration of the free or bound TRAF can then be detected and/or the dissociation constant of the I-TRAF/TRAF complex can be determined and compared with that of the control.

In order to identify lead compounds for therapeutically active agents that mimic the biological activity of a native ligand of a TNF receptor superfamily member with which a TRAF protein is associated (e.g. TNF, CD40 ligand, CD30 ligand, etc.), the candidate agent is added to a mixture of I-TRAF and TRAF (e.g. TRAF2 or TRAF3). The mutual ratio of the TRAF protein and the cellular protein and the incubation conditions are selected such that I-TRAF/TRAF complexes are formed prior to the addition of the candidate agent. Upon addition of a candidate agent, its ability to release TRAF from the I-TRAF/TRAF complex is tested. The typical assay conditions, e.g. incubation temperature, time, separation and detection of bound and unbound material, etc. are as hereinabove described. In a particular version of this assay, the assay mixture additionally contains a cellular protein with which the TRAF is normally associated (e.g. TNF-R2 or CD40), and the mutual ratio of the TRAF protein and the cellular protein and the incubation conditions are selected such that TRAF signaling does not occur prior to the addition of the candidate ligand analog. Upon addition of the candidate molecule, its ability to initiate a TRAF-mediated signaling event is detected. As an end point, it is possible to measure the ability of a candidate agent to induce TRAF-mediated NF-κB activation in a conventional manner. According to a preferred method, an NF-κB-dependent reporter gene, such as an E-selectin-luciferase reporter construct (Schindler and Baichwal, Mol. Cell. Biol. 14, 5820 [1994]), is used in a cell type assay. Luciferase activities are determined and normalized based on β-galactosidase expression. Alternatively, NF-κB activation can be analyzed by electrophoretic mobility shift assay (Schütze *et al.*, Cell 71, 765-776 [1992]). However, other conventional biochemical assays are equally be suitable for detecting the release of TRAF from its complexed (and inhibited) form.

Based upon their ability to bind TRAFs, the I-TRAF polypeptides of the present invention can be used to purify native and variant TRAFs and their functional derivatives, which, in turn, are useful for the purification of TNF-R2, CD40 and other members of the TNF receptor superfamily to which they specifically bind. Members of the TNF receptor superfamily are promising candidates for the treatment of a variety of pathological conditions, for example, TNF-R2 (either as a soluble protein or as a TNF-R2-Ig immunoadhesin) is in clinical trials for the treatment of endotoxic (septic shock) and rheumatoid arthritis (RA).

The I-TRAF molecules of the present invention may additionally be used to generate blocking (antagonist) or agonist anti-I-TRAF antibodies, which block or mimic the ability of I-TRAF to inhibit TRAF-mediated signal transduction. Generic methods for generating antibodies are well known in the art and are, for example, described in the textbooks and other literature referenced hereinbefore in connection with the definition of antibodies and concerning general techniques of recombinant DNA technology.

The I-TRAF molecules (including functional derivatives of native I-TRAFs) are also useful as therapeutics for the treatment of pathological conditions associated with downstream effects of the oncogene LMP1 on cell growth and gene expression. LMP1 is an Epstein-Barr virus transforming protein which is believed to play an important role in the development of tumors associated with EB virus, such as Burkitt's lymphoma (cancer of B lymphocytes) which has high incidence in West Africa and Papua New Guinea, and nasopharyngeal carcinoma, which is most common in Southern China and Greenland. Based upon their ability to block TRAF-mediated signaling through LMP1, the I-TRAFs of the present invention may inhibit the downstream effects of LMP1, including LMP1-mediated NF-κB activation, and are, optionally in combination with other cancer therapies, promising agents for the treatment of these types of cancers.

The *in vivo* efficacy of the treatment of the present invention can be studied against chemically induced tumors in various rodent models. Tumor cell lines propagated in *in vitro* cell cultures can be introduced in experimental rodents, e.g. mice by injection, for example by the subcutaneous route. Techniques for chemical inducement of tumors in experimental animals are well known in the art.

The treatment of the present invention may be combined with known tumor therapies, such as radiation therapy, chemotherapy, and immunotoxin therapy.

Therapeutic compositions comprising the I-TRAF polypeptides of the present invention, including functional derivatives, are prepared for storage by mixing the active ingredient having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th Edition, Osol, A. Ed. 1980) in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone, amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronics or PEG.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsulesand poly-(methylmethacylate)microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles

and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, *supra*.

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution.

Therapeutic compositions herein generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

The route of administration is in accord with known methods, e.g. injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial or intralesional routes, topical administration, or by sustained release systems.

Suitable examples of sustained release preparations include semipermeable polymer matrices in the form of shaped articles, e.g. films, or microcapsules. Sustained release matrices include polyesters, hydrogels, polylactides (U.S. Patent 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (U. Sidman *et al.*, Biopolymers 22 (1): 547-556 [1983]), poly (2-hydroxyethyl-methacrylate) (R. Langer, et al., J. Biomed. Mater. Res. 15: 167-277 [1981] and R. Langer, Chem. Tech. 12: 98-105 [1982]), ethylene vinyl acetate (R. Langer et al., Id.) or poly-D-(-)-3-hydroxybutyric acid (EP 133,988). Sustained release compositions also include liposomes. Liposomes containing a molecule within the scope of the present invention are prepared by methods known per se: DE 3,218,121; Epstein *et al.*, Proc. Natl. Acad. Sci. USA 82: 3688-3692 (1985); Hwang *et al.*, Proc. Natl. Acad. Sci. USA 77: 4030-4034 (1980); EP 52322; EP 36676A; EP 88046; EP 143949; EP 142641; Japanese patent application 83-118008; U.S. patents 4,485,045 and 4,544,545; and EP 102,324. Ordinarily the liposomes are of the small (about 200-800 Angstroms)

unilamelar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal NT-4 therapy.

An effective amount of the active ingredient will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. A typical daily dosage might range from about 1 μg/kg to up to 100 mg/kg or more, depending on the factors mentioned above. Typically, the clinician will administer a molecule of the present invention until a dosage is reached that provides the required biological effect. The progress of this therapy is easily monitored by conventional assays.

Further details of the invention will be apparent from the following non-limiting examples.

Example 1

Cloning of murine and human I-TRAFs

I-TRAF was identified in a yeast two-hybrid screening system (Fields and Song, Nature 340, 245-246 [1989]) as described (Rothe *et al.*, Cell 78 681-692 [1994]), using full length TRAF2 in the GAL4 DNA-binding domain vector pPC97 (Chevray and Nathans, Proc. Natl. Acad. Sci. USA 89, 5789-5793 [1992]) as bait. A plasmid cDNA library in the GAL4 transcriptional activation domain vector pPC86 (Chevray and Nathans, *supra*) was constructed from *Sal*I-*Not*I-adapted, double-stranded fetal liver stromal cell line 7-4 cDNA (FL, Rothe *et al.*, *supra*) and murine peripheral lymph nodes (PLN; provided by P. Young, D. Dowbenko, and L. Lasky; U.S. Patent No. 5,304,640 issued April 19, 1994). Transformation efficiencies for the two libraries were 10 and 1 million, respectively. Restriction mapping of 24 positive clones indicated that most were derived from the same gene. Four FL and two PLN cDNA clones were sequenced on a 373A automated DNA sequencer (Applied Biosystems). The 5' ends of six additional murine I-TRAF DNA clones isolated by screening a CT6 cDNA library prepared in λgt22a were also sequenced (Rothe *et al.*, *supra*). The

isolated cDNAs corresponded to several distinct transcripts of the murine I-TRAF gene. Due to alternative splicing, these transcripts have the potential to utilize two different translation initiation codons. The two major forms of murine I-TRAF mRNA are predicted to encode proteins of 413 and 447 amino acids that we have termed I-TRAFα and I-TRAFβ, respectively (Figure 1a; SEQ. ID. NOs: 1 and 2).

Five human I-TRAF cDNA clones, obtained by screening a λgt11 HUVEC cDNA library (Hsu *et al.*, Cell 81, 495-504 [1995]) with a murine I-TRAF probe, were sequenced. All isolated human I-TRAF cDNAs encode a full length 425 amino acid protein (M_r 48 K) that shows a 82% amino acid identity to murine I-TRAFα (Figure 1a). Database searches failed to reveal any proteins having significant sequence similarity to I-TRAF. Northern blot analysis using mRNA from mouse tissues indicated that the ~2.4 kb I-TRAF mRNA is ubiquitously expressed (Figure 1b). The multiple tissue blot (Clontech) was hybridized with a murine I-TRAF cDNA probe according to the Clontech protocol. I-TRAF mRNA is the band at ~2.4 kb.

Murine and human I-TRAF cDNA sequences have been deposited in GenBank and in the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD, USA, under accession numbers and, respectively.

Example 2

Characterization of I-TRAF

(1) Two-hybrid binding assay

Inspection of the various I-TRAF cDNAs obtained by two-hybrid screening indicated that the N-terminal portion of murine I-TRAF (amino acids 35-236) is sufficient for interaction with TRAF2. Further two-hybrid experiments were performed to determine which portion of TRAF2 is required for interaction with I-TRAF. Yeast HF7c cells were cotransformed with an expression

vector encoding a Gal14 activation domain-I-TRAF fusion protein and the indicated Gal4 DNA-binding domain expression vectors (Rothe *et al., supra*). Each transformation mixture was plated on a synthetic dextrose plate lacking leucine and tryptophan. Filter assays for β-galactosidase activity were performed to detect interaction between fusion proteins (Fields and Song, *supra*). The data are presented in Table 1 below.

TABLE 1 Interactions between I-TRAF and TRAFs, TNF-R2

DNA-binding domain hybrid	Activation domain hybrid	Interaction*
-	I-TRAF	-
TRAF1	I-TRAF	++
TRAF2	I-TRAF	++
TRAF2(87-501)	I-TRAF	++
TRAF3	I-TRAF	+
TNF-R2	I-TRAF	-

* Double plus and plus signs indicate strong blue color development within 30 minutes and one hour of the assay, respectively. Minus sign indicates no development of color within 24 hours.

The results show that only the conserved TRAF domain (amino acids 264-501) of TRAF2 is required for interaction with I-TRAF. Furthermore, both TRAF1 and TRAF3 also associate with I-TRAF.

(2) *In vitro* binding assay

The interactions of ^{35}S-labeled TRAF1 (Rothe *et al., supra*), TRAF2 (Rothe *et al., supra*), TRAF3 (Hu *et al., supra*), TNF-R2 (Smith *et al.*, Science 248, 1019-1023 [1990]), and TRADD (Hsu *et al.*, Cell 81, 495-504 [1995]) with I-TRAFα expressed as a glutathione-S-transferase(GST) fusion protein (GST-I-TRAF) and control GST protein were examined. GST-I-TRAF was expressed

using the pGEX3X vector (Pharmacia) and purified as described (Smith and Johnson, Gene 67, 31-40 [1988]). ^{35}S-labeled proteins were generated using TNT coupled reticulocyte lysate system (Promega) and the various cDNAs cloned in pBluescript KS (Stratagene) or pRK5 (Schall *et al.*, Cell 61, 361-370 [1990]). For each binding assay, 0.5 μg GST-I-TRAF or 0.5 μg GST bound to glutathione Sepharose beads was incubated with equivalent cpm of the individual ^{35}S-labeled proteins in 1 ml of E1A buffer (50 mM HEPES [pH 7.6], 250 mM NaCl, 0.1% NP-40, 5 mM EDTA) at 4 oC for 1 hour. Beads were washed six times with E1A buffer and precipitates were fractionated by 10% SDS-PAGE. The gel was dried and exposed to Kodak X-ray film. By this assay, I-TRAF was found to specifically interact with TRAF1, TRAF2, and TRAF3.

Results from both two-hybrid and *in vitro* binding experiments show that I-TRAF interacts more strongly with TRAF1 and TRAF2 than with TRAF3.

(3) Three-hybrid interaction test

I-TRAF does not directly interact with TNF-R2 (Table 1). However, as TRAF1 and TRAF2 form a complex with TNF-R2, it was important to ask if I-TRAF can indirectly associate with TNF-R2 via TRAFs. A three-hybrid interaction test (Rothe *et al.*, *supra)* was performed to address this question. Whereas TRAF2 can bind simultaneously to TRAF1 and TNF-R2 (Rothe *et al.*, *supra*), it was not able to mediate I-TRAF interaction with TNF-R2. In fact, I-TRAF expression in yeast was found to inhibit the association of TRAF2 with TNF-R2 (data not shown). This result is consistent with I-TRAF and TNF-R2 both binding to the same C-terminal TRAF domain of TRAF2.

(4) Inhibition of TRAF2:TNF-R2 interaction in mammalian cells

The observed inhibitory effect of I-TRAF on the TRAF2:TNF-R2 interaction was further investigated in mammalian cells. TRAF2 and I-TRAF were expressed as full length proteins containing N-terminal FLAG epitope tags (Kodak) using the cytomegalovirus-based expression vector pRK5 (Schall *et al.*, *supra*). Human embryonic kidney 293 cells ($2x10^6$) were seeded on 150

mm dishes and transfected (Ausubel *et al.*, Curr. Prot. Mol. Biol. 1, 9.1.1-9.1.3 [1994]) the following day with pRK-TRAF2 (0 or 1μg) expression vector (Rothe *et al.*, Science *in press*; copending application Serial No. 08/446,915 filed 22 May 1995) and increasing amounts of pRK-FLAG-I-TRAF (0, 1, 3, or 9 μg) expression vector. Cells were incubated at 37°C for 24 hours, and lysed in 0.5 ml E1A buffer. 450 μl aliquots of the lysates were incubated with 10 μl GST-TNF-R2 fusion protein (Rothe *et al.*, Cell *supra*; copending application Serial No. 08/446,915) bound to glutathione Sepharose beads for 15 minutes at 4 °C. The beads were washed three times with 0.6 ml E1A buffer and bound proteins fractionated by 8% SDS-PAGE and transferred to a nitrocellulose membrane. Western blot analysis was performed using an anti-FLAG monoclonal antibody M2 (Kodak) and horseradish peroxidase-coupled rabbit anti-mouse immunoglobulin (Amersham). Detection was by enhanced chemiluminescence according to the Amersham protocol. Western blot analysis of 50 μl aliquots of the lysates indicated that TRAF2 expression levels were similar in all samples transfected with pRK-TRAF2 (data not shown). Coexpression of TRAF2 with increasing amounts of I-TRAF effectively blocked the TRAF2:TNF-R2 interaction (Figure 3). Furthermore, no I-TRAF was precipitated by the TRAF2:TNF-R2 complex. These results show that TRAF2 us not able to bind TNF-R2 and I-TRAF simultaneously, possibly due to binding sites that are overlapping.

The ability of I-TRAF to bind the three known TRAF family members raised the intriguing possibility that I-TRAF functions as a general regulator of TRAF-mediated signaling events. Furthermore, the observed inhibitor effect of I-TRAF on TRAF2:TNF-R2 interaction suggested that I-TRAF expression might negatively influence TRAF2 signal transduction. Consequently, we measured the effect of I-TRAF expression on TRAF2-mediated NF-κB activation.

(5) The inhibitory effect of I-TRAF on activation of NF-κB

Human embryonic kidney 293 cells were seeded at 2 x 10^5 cells/well on 6-well (35 mm) dishes and transfected (Ausubel *et al.*, Curr. Prot. Mol. Biol. 1, 9.1.1-0.1.3 [1994]) the following day with (a) 0.5 μg pRK-TRAF2 (Rothe *et al.*, Science *in press*; copending application Serial No.

08/446,915) (b) 1μg pCDM8-CD40 (Rothe *et al.*, Science *supra*; copending application Serial No. 08/446,915), or (c) 0.1μg pRK5mTNF-R2 (Lewis *et al.*, Proc. Natl. Acad. Sci. USA 88, 2830-2834 [1991]), and increasing amounts of pRK-I-TRAF (0, 0.1, 0.32, 1.0 and 3.0 μg). Each transfection also contained 0.25 μg of pELAM-luc reported plasmid and 1 μg pRSV-βgal (Hsu *et al., supra*). For each of (a), (b) and (c), a control transfection containing only the pELAM-luc and pRSV-βgal was performed. After 24 hours, luciferase activities and normalizations to β-galactosidase levels were performed as described (Hsu *et al.*, *supra*). Values relative to control are shown in Figure 4 as mean ± SEM for experiments in which each transfection was performed in triplicate.

Transient expression of TRAF2 in 293 cells potently activates the NF-κB-dependent reported gene. (Rothe *et al.*, Science *supra*). This activation was dramatically inhibited by I-TRAF coexpression in a dose-dependent manner (Figure 4a). We next examined the effect of I-TRAF overexpression on NF-κB activation triggered by the TRAF2-interacting receptors CD40 and TNF-R2. Transient expression of these receptors has been shown to induce ligand-independent receptor aggregation which activated NF-κB in a TRAF2-dependent process. (Rothe *et al.*, Science *supra*). As observed above for TRAF2, NF-κB activation through both CD40 (Figure 4b) and TNF-R2 (Figure 4c) was effectively blocked by increased expression of I-TRAF. NF-κB activation mediated by interleukin 1 (IL-1) was only slightly depressed by I-TRAF overexpression (data not shown). Thus, I-TRAF is a specific inhibitor of TRAF2-dependent NF-κB activation signaled by CD40 and TNF-R2.

(6) Conclusions

Conceptually, the I-TRAF/TRAF system bears remarkable similarity to the extensively studied IκB/NF-κB regulatory system (Liou and Baltimore, Curr. Op. Cell. Bio. 5, 477-487 [1993]; Thanos & Maniatis, Cell 80, 529-532 [1995]). Both systems are involved in regulating the activity of the transcription factor NF-κB through the utilization of inhibitory proteins. IκB directly regulates NF-κB function by keeping it sequestered in the cytoplasm. I-TRAF acts at an earlier step

by binding to TRAFs and preventing their interaction with cell surface receptors (CD40, TNF-R2) that trigger TRAF2-mediated NF-κB activation cascades. The function of I-TRAF may be to prevent continuous TRAF signaling in the absence of ligand (CD40L or TNF). This function is consistent with the finding that TRAF2 overexpression alone, in the absence of receptor aggregation, can trigger NF-κB activation. Ligand-induced receptor aggregation might be expected to release TRAFs from I-TRAF inhibition by providing a new, higher affinity TRAF binding site.

All documents cited throughout the specification and all references cited therein are hereby expressly incorporated by reference. Although the invention has been described with reference to certain embodiments, it will be understood that certain variations and modification are possible and will be readily available for those of ordinary skill in the art. All such changes and modification and with the scope of the present invention.

Claims:

1. An isolated I-TRAF polypeptide comprising the amino acid sequence of a polypeptide selected from the group consisting of:

(a) a native sequence I-TRAF polypeptide,

(b) a polypeptide having at least about 70% amino acid sequence identity with the TRAF-binding domain of a native sequence I-TRAF polypeptide and capable of inhibiting a TRAF-mediated signaling event, and

(c) a fragment of a polypeptide of (a) or (b) capable of inhibiting a TRAF-mediated signaling event.

2. The polypeptide of claim 1 which has an at least about 60% overall amino acid sequence identity with a native sequence I-TRAF polypeptide.

3. The polypeptide of claim 1 which has an at least about 70% overall amino acid sequence identity with a native sequence I-TRAF polypeptide.

4. The polypeptide of claim 1 which has an at least about 80% overall amino acid sequence identity with a native sequence I-TRAF polypeptide.

5. The polypeptide of claim 1 which has an at least about 90% overall amino acid sequence identity with a native sequence I-TRAF polypeptide.

6. The polypeptide of claim 1, which is a native mammalian I-TRAF or a fragment thereof.

7. The polypeptide of claim 6 which is a native murine I-TRAF or a fragment thereof.

8. The polypeptide of claim 7 comprising amino acids 35 - 236 of SEQ. ID. NO 1.

9. The polypeptide of claim 6 which is a native human I-TRAF or a fragment thereof.

10. The polypeptide of claim 9 comprising amino acids 1 through 202 of SEQ. ID. NO: 5.

11. The polypeptide of claim 9 represented by SEQ. ID. NO: 5.

12. The polypeptide of claim 9 encoded by a nucleic acid capable of hybridizing under stringent conditions to the complement of SEQ. ID. NO: 6.

13. An isolated nucleic acid molecule encoding a polypeptide of claim 1.

14. An isolated nucleic acid molecule encoding a polypeptide of claim 9.

15. A vector comprising and capable of expressing, in a suitable host cell,. the nucleic acid molecule of claim 13.

16. A vector comprising and capable of expressing, in a suitable host cell, the nucleic acid molecule of claim 14.

17. A host cell transformed with the nucleic acid molecule of claim 13.

18. A host cell transformed with the nucleic acid molecule of claim 14.

19. An antibody capable of specific binding to an I-TRAF polypeptide of claim 1.

20. A hybridoma cell line producing an antibody of claim 19.

21. A method of using a nucleic acid molecule of claim 13 comprising expressing such nucleic acid molecule in a cultured host cell transformed therewith, and recovering the polypeptide encoded by said nucleic acid molecule from the host cell.

22. A method of producing an I-TRAF polypeptide of claim 1 comprising inserting into the DNA of a cell containing nucleic acid encoding said polypeptide a transcription modulatory element in sufficient proximity and orientation to the nucleic acid molecule to influence the transcription thereof.

23. An assay for identifying a molecule capable of modulating the association of an I-TRAF and a TRAF comprising (a) incubating a mixture comprising said I-TRAF and TRAF with a candidate molecule and (b) detecting of the ability of the candidate molecule to modulate I-TRAF/TRAF association.

24. The assay of claim 23 wherein said candidate molecule prevents or inhibits the dissociation of an I-TRAF/TRAF complex.

25. The assay of claim 23 wherein said candidate molecule prevents the association of I-TRAF and TRAF.

26. The assay of claim 23 wherein said candidate molecule is a small molecule.

27. An assay for identifying a molecule the signal transduction of which is mediated by the association of a TRAF with a cellular protein, comprising (a) incubating a mixture comprising

said TRAF and an I-TRAF with a candidate molecule, and (b) detecting the ability of said candidate molecule to release TRAF from a complex formed with I-TRAF.

28. The assay of claim 27 wherein said mixture further comprises said cellular protein, and the ability of said candidate molecule to initiate TRAF-mediated signaling through said cellular protein is detected.

29. The assay of claim 23 or claim 28 wherein said TRAF comprises the TRAF domain of a native TRAF1, TRAF2 or TRAF3 polypeptide.

30. The assay of claim 23 or claim 28 wherein said TRAF, I-TRAF and cellular protein are human.

31. The assay of claim 30 wherein said TRAF comprises amino acids 264-501 of a native human TRAF2 polypeptide.

32. The assay of claim 30 wherein said I-TRAF comprises the N-terminal portion of a native human I-TRAF polypeptide.

33. The assay of claim 28 wherein said cellular protein is a member of the TNF receptor superfamily.

34. The assay of claim 33 wherein said cellular protein is selected from the group consisting of TNF-R2, CD40 and CD30.

35. A method of treating a tumor associated with Epstein-Barr virus comprising administering to a patient having developed or at risk of developing such tumor a therapeutically effective amount of an I-TRAF. .

36. The method of claim 35 wherein said tumor is selected from the group of Burkitt's lymphoma and nasopharyngal carcinoma.

37. A pharmaceutical formulation comprising a therapeutically effective amount of an I-TRAF in association with a pharmaceutically acceptable carrier.

38. A method of modulating TRAF-mediated signal transduction in a cell comprising introducing into said cell a nucleic acid according to claim 13 whereby said nucleic acid is expressed in said cell and the resultant gene product modulates TRAF-mediated signal transduction in said cell.

Abstract of the Disclosure

The invention concerns novel inhibitors of tumor necrosis factor receptor associated factor-(TRAF) mediated signal transduction. The invention encompasses the novel inhibitor proteins (I-TRAFs), nucleic acid encoding them, methods for their recombinant production, and their use in screening assays and as pharmaceuticals.

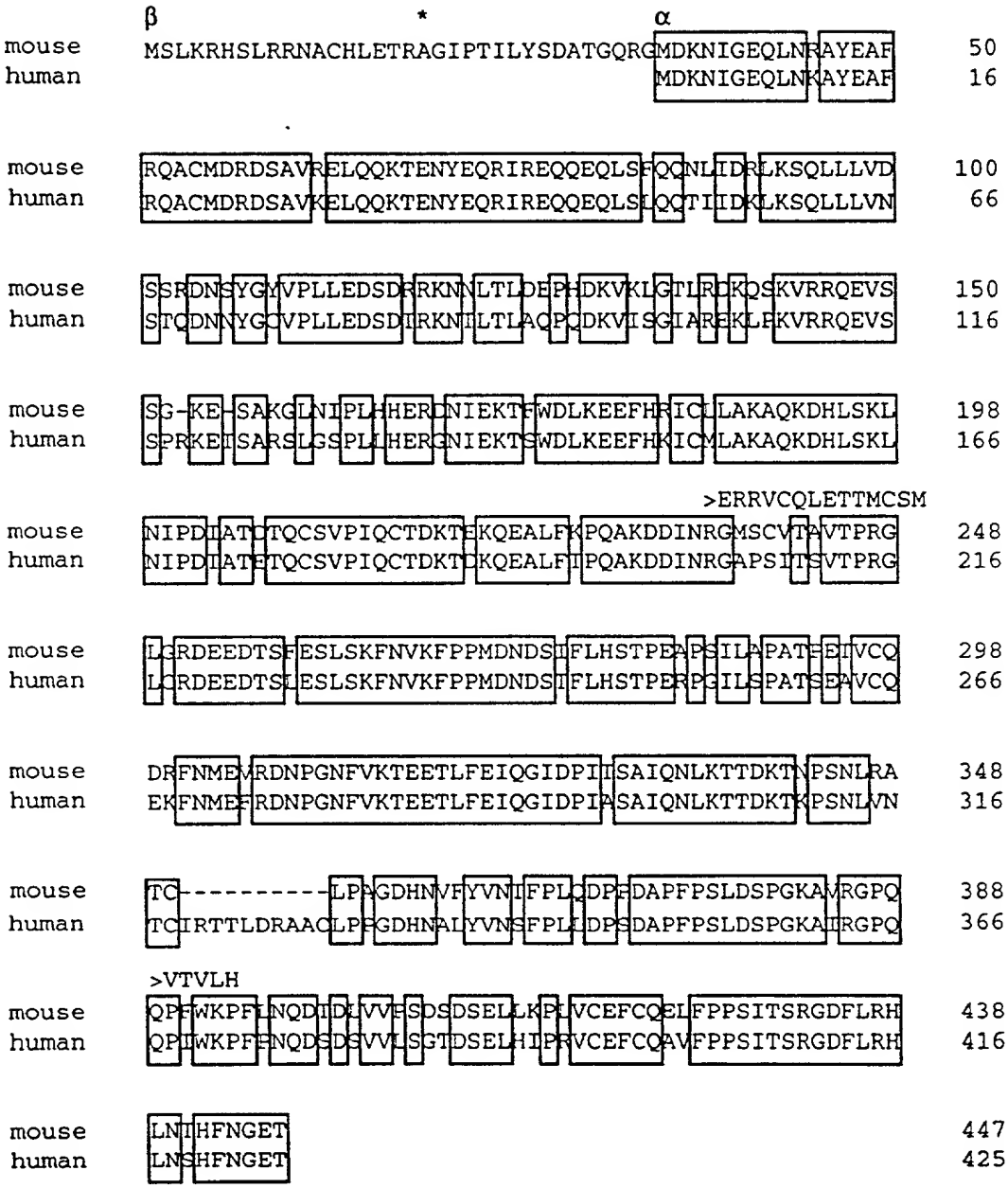

β
*
α
mouse MSLKRHSLRRNACHLETRAGIPTILYSDATGQRGMDKNIGEQLNRAYEAF 50
human MDKNIGEQLNKAYEAF 16
mouse RQACMDRDSAVRELQQKTENYEQRIREQQEQLSFQQNLIDRLKSQLLLVD 100
human RQACMDRDSAVKELQQKTENYEQRIREQQEQLSLQQTIIDKLKSQLLLVN 66
mouse SSRDNSYGMVPLLEDSDRRKNNLTLDEPHDKVKLGTLRDKQSKVRRQEVS 150
human STQDNNYGQVPLLEDSDIRKNILTLAQPQDKVISGIARELKKVRRQEVS 116
mouse SG-KE-SAKGLNIPLHHERLNIEKTFWDLKEEFHRICLLAKAQKDHLSKL 198
human SPRKETSARSLGSPLLHERGNIEKTSWDLKEEFHKICMLAKAQKDHLSKL 166
>ERRVCQLETTMCSM
mouse NIPDIATDTQCSVPIQCTDKTEKQEALFKPQAKDDINRGMSCVIAVTPRG 248
human NIPDIATETQCSVPIQCTDKTDKQEALFTPQAKDDINRGAPSIITSVTPRG 216
mouse LGRDEEDTSFESLSKFNVKFPPMDNDSIFLHSTPEAPSILAPATEEIVCQ 298
human LGRDEEDTSLESLSKFNVKFPPMDNDSTFLHSTPERPGILSPATSEAVCQ 266
mouse DRFNMEMRDNPGNFVKTEETLFEIQGIDPIISAIQNLKTTDKTNPSNLRA 348
human EKFNMEFRDNPGNFVKTEETLFEIQGIDPIASAIQNLKTTDKTKPSNLVN 316
mouse TC----------LPAGDHNVFYVNTFPLQDPEDAPFPSLDSPGKAMRGPQ 388
human TCIRTTLDRAACLPPGDHNALYVNSFPLLDPSDAPFPSLDSPGKAIRGPQ 366
>VTVLH
mouse QPFWKPFLNQDIDLVVESDSDSELLKPLVCEFCQELFPPSITSRGDFLRH 438
human QPIWKPFFNQDSDSVVLSGTDSELHIPRVCEFCQAVFPPSITSRGDFLRH 416
mouse LNIHFNGET 447
human LNSHFNGET 425

Figure 1a

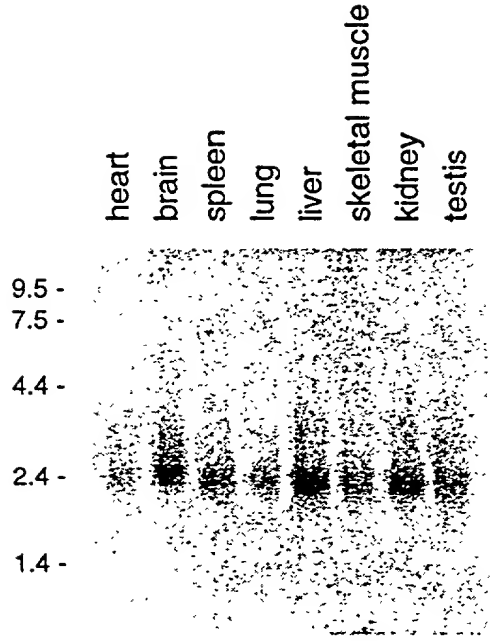
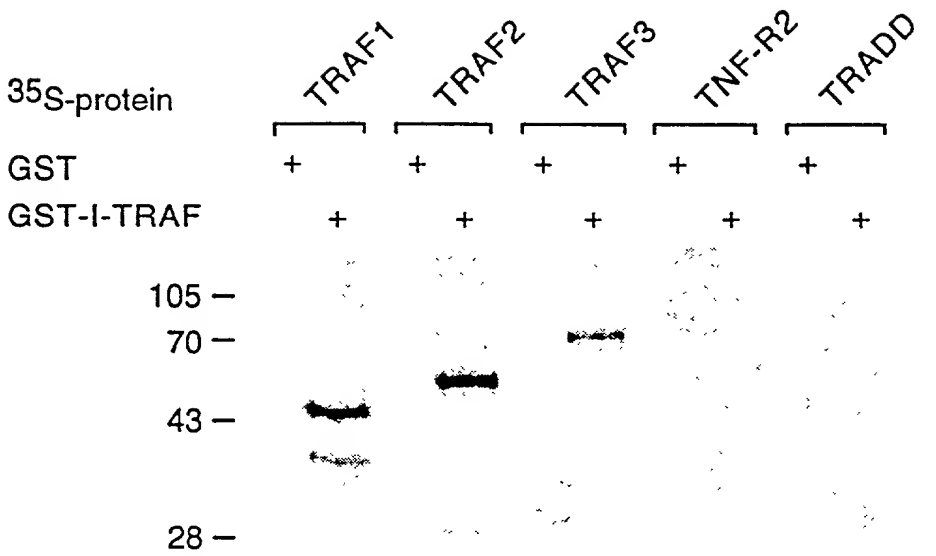

heart
brain
spleen
lung
liver
skeletal muscle
kidney
testis
9.5 -
7.5 -
4.4 -
2.4 -
1.4 -

Figure 1b


35S-protein
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TRAF2
TRAF3
TNF-R2
TRADD
GST
GST-I-TRAF
105
70
43
28

Figure 2

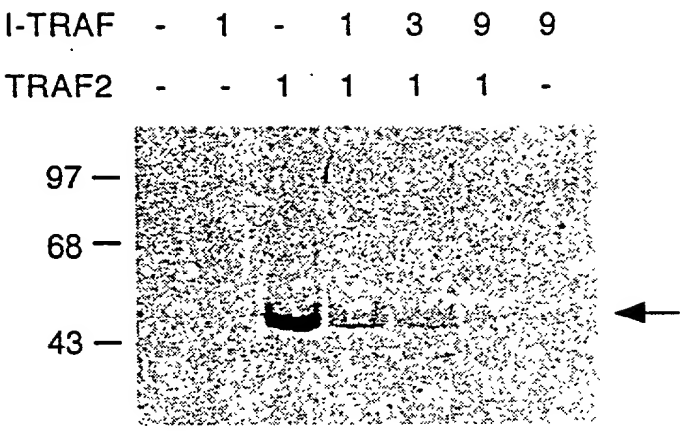

Figure 3

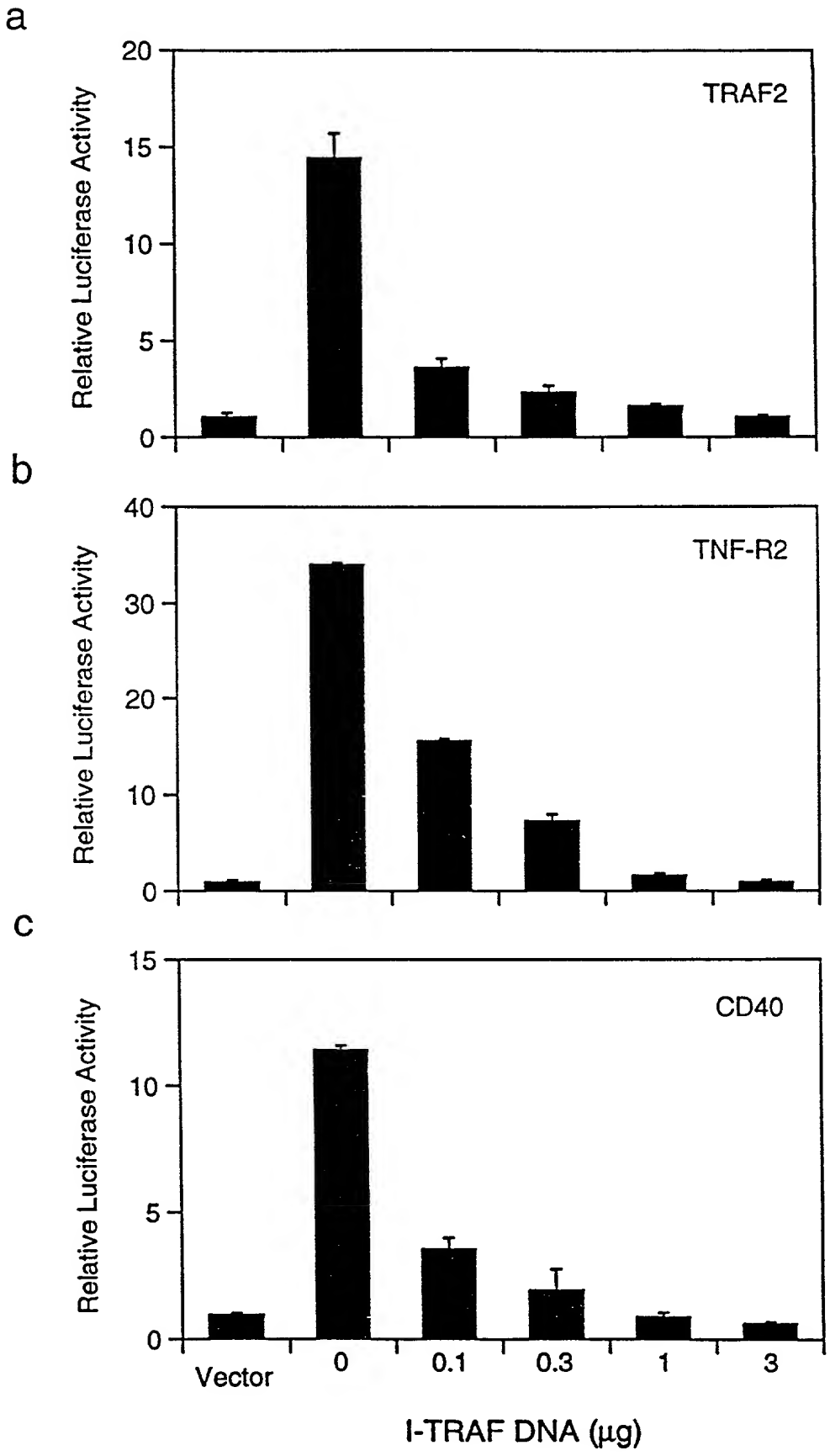
a
b
c
TRAF2
TNF-R2
CD40
Relative Luciferase Activity
20
15
10
5
0
40
30
20
10
0
15
10
5
0
Vector
0
0.1
0.3
1
3
I-TRAF DNA (μg)

Figure 4

EXPRESS MAIL NO: GB 590 945 520 US
DATED: 15 August 1996
Docket No. P0960R1

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

TRAF INHIBITORS

the specification of which (check one) X is attached hereto or _ was filed on _ as Application Serial No. _ and was amended on _ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate have a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s):			Priority Claimed Yes	Priority Claimed No
Number	Country	Day/Month/Year Filed		

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional applications(s) listed below:

60/002382	17 August 1995
Application Ser. No.	Filing Date

I hereby claim the benefit under Title 35, United States Code, §120 of any United States applications(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Ser. No.	Filing Date	Status: Patented, Pending, Abandoned
Application Ser. No.	Filing Date	Status: Patented, Pending, Abandoned

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Walter E. Buting - Reg. No. 23,092	Jeffrey S. Kubinec - Reg. No. 36,575
Ginger R. Dreger - Reg. No. 33,055	Stephen Raines - Reg. No. 25,912
Renee A. Fitts - Reg. No. 35,136	Richard B. Love - Reg. No. 34,659
Janet E. Hasak - Reg. No. 28,616	Diane L. Marschang - Reg. No. 35,600
Sean A. Johnston - Reg. No. 35,910	Daryl B. Winter - Reg. No. 32,637
Dennis G. Kleid - Reg. No. 32,037	Timothy E. Torchia - Reg. No. 36,700

Send correspondence to

Genentech, Inc.
Attn: Ginger R. Dreger
460 Point San Bruno Boulevard
South San Francisco, CA 94080
Telephone: (415) 225-3216

I hereby declare that all statements made herein of my own knowledge and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that willful false statements may jeopardize the validity of the application or any patent issued thereon.

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from his foreign patent agent as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

Full name of sole or first inventor

David V. Goeddel

Inventor's signature

Date 7/28/96

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Full name of second joint inventor, if any

Mike Rothe

Inventor's signature

Date 7/26/1996

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South San Francisco, CA 94080

Full name of third joint inventor, if any

Inventor's signature

Date

Residence

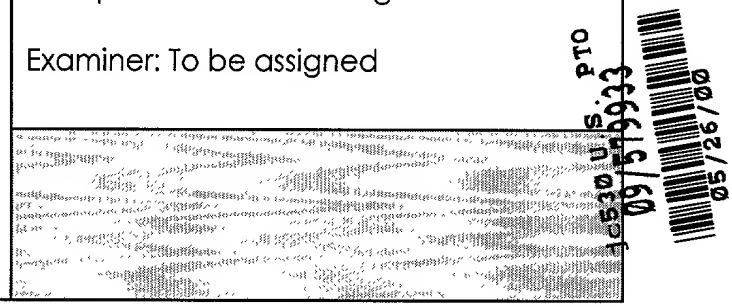
Citizenship

Post Office Address
460 Point San Bruno Boulevard
South San Francisco, CA 94080

Patent Docket P0960R1D1C2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Goeddel et al. Serial No.: To be assigned Filed: May 26, 2000 For: TRAF INHIBITORS	Group Art Unit: To be assigned Examiner: To be assigned



CERTIFICATE REGARDING SEQUENCE LISTING

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

I hereby state that the information recorded in computer readable form in accordance with 37 CFR Section 1.821 is identical to the paper copy of the Sequence Listing provided herewith.

Respectfully submitted,
GENENTECH, INC.

Date: May 26, 2000

By: Diane L Marschang
Diane L. Marschang
Reg. No. 35,600

1 DNA Way
So. San Francisco, CA 94080-4990
Phone: (650) 225-5416
Fax: (650) 952-9881

Patent Docket P0960R1D1C2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Goeddel et al. Serial No.: To be assigned Filed: May 26, 2000 For: TRAF INHIBITORS	Group Art Unit: To be assigned Examiner: To be assigned

REQUEST TO USE COMPUTER-READABLE SEQUENCE LISTING

UNDER 37 CFR §1.821(e)

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Applicants respectfully request that the compliant computer-readable Sequence Listing filed in prior application Serial No. 09/020,467 (to which priority is claimed under Section 120) be used as the computer-readable Sequence Listing for the present, above-identified application.

The paper copy of the Sequence Listing filed in the present application is identical to the computer-readable copy of the Sequence Listing filed in the application Serial No. 09/020,467.

Respectfully submitted,
GENENTECH, INC.

Date: May 26, 2000

By: Diane L Marschang
Diane L. Marschang
Reg. No. 35,600

1 DNA Way
So. San Francisco, CA 94080-4990
Phone: (650) 225-5416
Fax: (650) 952-9881

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: GOEDDEL, DAVID V.
ROTHE, MIKE

(ii) TITLE OF INVENTION: TRAF INHIBITORS

(iii) NUMBER OF SEQUENCES: 8

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Genentech, Inc.
(B) STREET: 1 DNA Way
(C) CITY: South San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94080

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WinPatin (Genentech)

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 60/002382

(B) FILING DATE: 17-aug-1995

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Dreger, Ginger R.

(B) REGISTRATION NUMBER: 33,055

(C) REFERENCE/DOCKET NUMBER: P0960R1D1

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 650/225-3216

(B) TELEFAX: 650/952-9881

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 413 amino acids

(B) TYPE: Amino Acid

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Asp Lys Asn Ile Gly Glu Gln Leu Asn Arg Ala Tyr Glu Ala
1 5 10 15

Phe Arg Gln Ala Cys Met Asp Arg Asp Ser Ala Val Arg Glu Leu
20 25 30

Gln Gln Lys Thr Glu Asn Tyr Glu Gln Arg Ile Arg Glu Gln Gln
35 40 45

Glu Gln Leu Ser Phe Gln Gln Asn Leu Ile Asp Arg Leu Lys Ser
50 55 60

Gln Leu Leu Leu Val Asp Ser Ser Arg Asp Asn Ser Tyr Gly Tyr
65 70 75

Val Pro Leu Leu Glu Asp Ser Asp Arg Arg Lys Asn Asn Leu Thr
80 85 90

Leu Asp Glu Pro His Asp Lys Val Lys Leu Gly Thr Leu Arg Asp
95 100 105

Lys Gln Ser Lys Val Arg Arg Gln Glu Val Ser Ser Gly Lys Glu
110 115 120

Ser Ala Lys Gly Leu Asn Ile Pro Leu His His Glu Arg Asp Asn
125 130 135

Ile Glu Lys Thr Phe Trp Asp Leu Lys Glu Glu Phe His Arg Ile
140 145 150

Cys Leu Leu Ala Lys Ala Gln Lys Asp His Leu Ser Lys Leu Asn
155 160 165

Ile Pro Asp Ile Ala Thr Asp Thr Gln Cys Ser Val Pro Ile Gln
170 175 180

Cys Thr Asp Lys Thr Glu Lys Gln Glu Ala Leu Phe Lys Pro Gln
185 190 195

Ala Lys Asp Asp Ile Asn Arg Gly Met Ser Cys Val Thr Ala Val
200 205 210

Thr Pro Arg Gly Leu Gly Arg Asp Glu Glu Asp Thr Ser Phe Glu
215 220 225

Ser Leu Ser Lys Phe Asn Val Lys Phe Pro Pro Met Asp Asn Asp
230 235 240

Ser Ile Phe Leu His Ser Thr Pro Glu Ala Pro Ser Ile Leu Ala
245 250 255

Pro Ala Thr Pro Glu Thr Val Cys Gln Asp Arg Phe Asn Met Glu
260 265 270

Val Arg Asp Asn Pro Gly Asn Phe Val Lys Thr Glu Glu Thr Leu
275 280 285

Phe Glu Ile Gln Gly Ile Asp Pro Ile Thr Ser Ala Ile Gln Asn
290 295 300

Leu Lys Thr Thr Asp Lys Thr Asn Pro Ser Asn Leu Arg Ala Thr
305 310 315

Cys Leu Pro Ala Gly Asp His Asn Val Phe Tyr Val Asn Thr Phe
320 325 330

Pro Leu Gln Asp Pro Pro Asp Ala Pro Phe Pro Ser Leu Asp Ser
335 340 345

Pro Gly Lys Ala Val Arg Gly Pro Gln Gln Pro Phe Trp Lys Pro
350 355 360

Phe Leu Asn Gln Asp Thr Asp Leu Val Val Pro Ser Asp Ser Asp
365 370 375

Ser Glu Leu Leu Lys Pro Leu Val Cys Glu Phe Cys Gln Glu Leu
380 385 390

Phe Pro Pro Ser Ile Thr Ser Arg Gly Asp Phe Leu Arg His Leu
395 400 405

Asn Thr His Phe Asn Gly Glu Thr
410 413

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1955 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTGGAACGGA AAGCTACTTC CGGTTGCAGT CATTCTGCCG GGCACCGGCG 50

ACCTGTGGCG TGAGCGAGCA CAGCCGGAAC CCTCCACTAG CTGGCATTCC 100

TACCATCCTT TATAGTGATG CTACAGGACA AAGAGGAATG GATAAAAACA 150

TTGGTGAGCA ACTCAATAGA GCATATGAAG CCTTCCGACA GGCATGCATG 200

GATAGAGATT CAGCAGTAAG AGAGCTACAG CAAAAGCAGA CTGAGAACTA 250

TGAACAAAGA ATACGCGAGC AACAGGAACA GCTGTCATTT CAACAAAACC 300

TAATTGACAG GCTGAAATCA CAGCTACTTC TCGTGGATTC TAGTCGAGAT 350

AACAGTTATG GCTATGTACC TTTGCTTGAA GACAGTGACA GAAGGAAGAA 400

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TCCGCCAAGG GTCTCAACAT CCCTCTGCAT CACGAAAGGG ATAATATAGA 550

GAAGACTTTC TGGGACCTTA AAGAAGAATT TCATAGGATT TGCTTGCTAG 600

CAAAAGCACA GAAAGATCAC TTAAGCAAAC TTAATATACC AGATATTGCA 650

ACTGACACAC AGTGTTCTGT GCCTATACAG TGTACTGATA AAACAGAGAA 700

ACAAGAAGCG CTGTTTAAGC CCCAGGCTAA AGATGATATA AATAGAGGTA 750

TGTCGTGCGT CACAGCTGTC ACACCAAGAG GACTGGGCCG GGATGAGGAA 800

GATACCTCTT TTGAATCACT TTCTAAATTC AATGTCAAGT TTCCGCCTAT 850

GGACAATGAC TCTATTTTTC TACATAGCAC TCCAGAGGCC CCGAGCATCC 900

TTGCTCCTGC CACACCTGAG ACAGTGTGCC AGGACCGATT TAATATGGAA 950

GTCAGAGACA ACCCAGGAAA CTTTGTTAAA ACAGAAGAAA CTTTATTTGA 1000

AATTCAGGGA ATTGACCCCA TAACTTCAGC TATACAAAAC CTTAAAACAA 1050

CTGACAAAAC AAACCCCTCA AATCTTAGAG CGACGTGTTT GCCAGCTGGA 1100

GACCACAATG TGTTCTATGT AAATACGTTC CCACTTCAAG ACCCGCCTGA 1150

CGCACCTTTT CCCTCACTGG ATTCCCCAGG AAAGGCTGTC CGAGGACCAC 1200

AGCAGCCCTT TTGGAAGCCT TTTCTTAACC AAGACACTGA CTTAGTGGTA 1250

CCAAGTGATT CAGACTCAGA GCTCCTTAAA CCTCTAGTGT GTGAATTCTG 1300

TCAAGAGCTT TTCCCACCAT CCATTACATC CAGAGGGGAT TTCCTCCGGC 1350

ATCTTAATAC ACACTTTAAT GGGGAGACTT AAATCACGTT TGAAAACAGA 1400

CATATCATGT TCTCTGTGGT GGTTTTGGAT TTGTAACGCT AGAGAACGCT 1450

TTCTCGTGAG CCAAATGTAA GATTGATTAT AAAGTTGTTA CTTTATCTTT 1500

TAAGAGATCA TTTTGTATAG AACTATAACT CATTATATTA TTCATGTTTA 1550

TACCTATAAT TTCTACATTT CAAAATTACA CATGTGACTT ACAGAGTTAT 1600

TCAGTCATAA TTTATGTTTC AAATAGCTAA GTTTATTGTT TGACTATTGT 1650

GAGATCTATT AAATTTAGTA ATAGCAAATG TTTATAGGAT ATTCAAATTT 1700

CATTTGAATT TTTAATTATT TTTGCTACAG GTAATATTCC TTTAAAATAC 1750

GTATATAACG TACAGAGAAT AACAGACAAT ATGATCTAAG TAAATGTCGA 1800

ATCAATCATT AGTTGCCCAG GGAAATTTAA ACATTATAGA TCATTTTTAA 1850

ATAATACACA TAGTTTTAAT TTTTACTGTG TGTATAGATG CATGATTAAA 1900

TGACTTAAAT ATTAAAAGTG ACTTACGTCG TGCTTATTAA AAAAAAAAAA 1950

AAAAA 1955

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 34 amino acids

(B) TYPE: Amino Acid

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Ser Leu Lys Arg His Ser Leu Arg Arg Asn Ala Cys His Leu
1 5 10 15

Glu Thr Arg Ala Gly Ile Pro Thr Ile Leu Tyr Ser Asp Ala Thr
20 25 30

Gly Gln Arg Gly
34

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 170 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCCACGCGTC CGGTTTGGGC AGCATCTGTA GAGCCTGTGC AAACGGCTTC 50

CAGAATGGGT ACGTGCCTAT GTCTTTAAAG AGACATAGTC TGCGAAGGAA 100

CGCCTGTCAC CTGGAGACGA GAGCTGGCAT TCCTACCATC CTTTATAGTG 150

ATGCTACAGG ACAAAGAGGA 170

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 425 amino acids

(B) TYPE: Amino Acid

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Met Asp Lys Asn Ile Gly Glu Gln Leu Asn Lys Ala Tyr Glu Ala
 1               5                  10                  15

Phe Arg Gln Ala Cys Met Asp Arg Asp Ser Ala Val Lys Glu Leu
            20                  25                  30

Gln Gln Lys Thr Glu Asn Tyr Glu Gln Arg Ile Arg Glu Gln Gln
        35                  40                  45

Glu Gln Leu Ser Leu Gln Gln Thr Ile Ile Asp Lys Leu Lys Ser
    50                  55                  60

Gln Leu Leu Leu Val Asn Ser Thr Gln Asp Asn Asn Tyr Gly Cys
65                  70                  75

Val Pro Leu Leu Glu Asp Ser Asp Thr Arg Lys Asn Thr Leu Thr
                80                  85                  90

Leu Ala Gln Pro Gln Asp Lys Val Ile Ser Gly Ile Ala Arg Glu
            95                  100                 105

Lys Leu Pro Lys Val Arg Arg Gln Glu Val Ser Ser Pro Arg Lys
        110                 115                 120
```

Glu Thr Ser Ala Arg Ser Leu Gly Ser Pro Leu Leu His Glu Arg
125 130 135

Gly Asn Ile Glu Lys Thr Ser Trp Asp Leu Lys Glu Glu Phe His
140 145 150

Lys Ile Cys Met Leu Ala Lys Ala Gln Lys Asp His Leu Ser Lys
155 160 165

Leu Asn Ile Pro Asp Thr Ala Thr Glu Thr Gln Cys Ser Val Pro
170 175 180

Ile Gln Cys Thr Asp Lys Thr Asp Lys Gln Glu Ala Leu Phe Thr
185 190 195

Pro Gln Ala Lys Asp Asp Ile Asn Arg Gly Ala Pro Ser Ile Thr
200 205 210

Ser Val Thr Pro Arg Gly Leu Cys Arg Asp Glu Glu Asp Thr Ser
215 220 225

Leu Glu Ser Leu Ser Lys Phe Asn Val Lys Phe Pro Pro Met Asp
230 235 240

Asn Asp Ser Thr Phe Leu His Ser Thr Pro Glu Arg Pro Gly Ile
245 250 255

```
Leu Ser Pro Ala Thr Ser Glu Ala Val Cys Gln Glu Lys Phe Asn
                260                 265                 270

Met Glu Phe Arg Asp Asn Pro Gly Asn Phe Val Lys Thr Glu Glu
                275                 280                 285

Thr Leu Phe Glu Ile Gln Gly Ile Asp Pro Ile Ala Ser Ala Ile
                290                 295                 300

Gln Asn Leu Lys Thr Thr Asp Lys Thr Lys Pro Ser Asn Leu Val
                305                 310                 315

Asn Thr Cys Ile Arg Thr Thr Leu Asp Arg Ala Ala Cys Leu Pro
                320                 325                 330

Pro Gly Asp His Asn Ala Leu Tyr Val Asn Ser Phe Pro Leu Leu
                335                 340                 345

Asp Pro Ser Asp Ala Pro Phe Pro Ser Leu Asp Ser Pro Gly Lys
                350                 355                 360

Ala Ile Arg Gly Pro Gln Gln Pro Ile Trp Lys Pro Phe Pro Asn
                365                 370                 375

Gln Asp Ser Asp Ser Val Val Leu Ser Gly Thr Asp Ser Glu Leu
                380                 385                 390
```

His Ile Pro Arg Val Cys Glu Phe Cys Gln Ala Val Phe Pro Pro
395 400 405

Ser Ile Thr Ser Arg Gly Asp Phe Leu Arg His Leu Asn Ser His
410 415 420

Phe Asn Gly Glu Thr
425

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2108 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GTTTGAGCAG CATTGTTAGA GCCTGTGGAA AACACTTTAC AACTGTGTAA 50

CTGTCTTCAT CTTTACAGAG GAATAGTCTA CAAAGGAAGA CTTGTAACCT 100

GGAGAAGAGA CCTGTCATTT ACTCCATCCT TTATAGTGAT GCTACAGGAC 150

GAAGAGGAAT GGATAAAAAC ATTGGCGAGC AACTCAATAA AGCGTATGAA 200

GCCTTCCGGC AGGCATGCAT GGATAGAGAT TCTGCAGTAA AAGAATTACA 250

GCAAAAGACT GAGAACTATG AGCAGAGAAT ACGTGAACAA CAGGAACAGC 300

TGTCACTCCA ACAGACTATT ATTGACAAGC TAAAATCTCA GTTACTTCTT 350

GTGAATTCCA CTCAAGATAA CAATTATGGC TGTGTCCCTC TGCTTGAAGA 400

CAGTGACACA AGAAAGAATA CTTTGACTCT TGCTCAGCCA CAAGATAAAG 450

TGATTTCAGG AATAGCAAGA GAAAAACTAC CAAAGGTAAG AAGACAAGAG 500

GTTTCTTCTC CTAGAAAAGA AACTTCAGCA AGGAGTCTTG GCAGTCCTTT 550

GCTCCATGAA AGGGGTAATA TAGAGAAGAC TTCCTGGGAT CTGAAAGAAG 600

AATTTCATAA AATATGCATG CTAGCAAAAG CACAGAAAGA CCACTTAAGC 650

AAACTTAATA TACCAGACAC TGCAACTGAA ACACAGTGCT CTGTGCCTAT 700

ACAGTGTACG GATAAAAACAG ATAAACAAGA AGCGCTGTTT ACGCCTCAGG 750

CTAAAGATGA TATAAATAGA GGTGCACCAT CCATCACATC TGTCACACCA 800

AGAGGACTGT GCAGAGATGA GGAAGACACC TCTTTGGAAT CACTTTCTAA 850

ATTCAATGTC AAGTTTCCAC CTATGGACAA TGACTCAACT TTCTTACATA 900

GCACTCCAGA GAGACCCGGC ATCCTTAGTC CTGCCACGTC TGAGGCAGTG 950

TGCCAAGAGA AATTTAATAT GGAGTTCAGA GACAACCCAG GGAACTTTGT 1000

TAAAACAGAA GAAACTTTAT TTGAAATTCA GGGAATTGAC CCCATAGCTT 1050

CAGCTATACA AAACCTTAAA ACAACTGACA AAACAAAGCC CTCAAATCTC 1100

GTAAACACTT GTATCAGGAC AACTCTGGAT AGAGCTGCGT GTTTGCCACC 1150

TGGAGACCAT AATGCATTAT ATGTAAATAG CTTCCCACTT CTGGACCCAT 1200

CTGATGCACC TTTTCCCTCA CTCGATTCCC CGGGAAAAGC AATCCGAGGA 1250

CCACAGCAGC CCATTTGGAA GCCCTTTCCT AATCAAGACA GTGACTCGGT 1300

GGTACTAAGT GGCACAGACT CAGAACTGCA TATACCTCGA GTATGTGAAT 1350

TCTGTCAAGC AGTTTTCCCA CCATCCATTA CATCCAGGGG GGATTTCCTT 1400

CGGCATCTTA ATTCACACTT CAATGGAGAG ACTTAAGACA CATTTGAAAA 1450

CAGACATATC AAGTTCTATG TGATGATTTT GGGTTTTTAA TACTATAAAT 1500

ACTTGATTGT AAACTAAATT CAAGATCATT TATAGGAAAA TCTAGTTTCA 1550

CAGCTATTTG AATTTTTTTC TGGATTTACT ATATAACTCT TATTTTTTAA 1600

AAGATCATTC TGTTCTTTCA AGGAGAAATA AGCCTAAAAG AAGAAAAACA 1650

AAAAAAATTC TGTATAAAAC TGTAATCCTT TGTATTCATG TTTACAGTGC 1700

TATTACTATA ATTCAAAATT ATGTATGTGA CTTAGAGTTA TATAATCATA 1750

ATTTATGTTT ATTTCAAATA TCTAAGTTTA TTGCTTGGAT TTCTAGTGAG 1800

AGCTGTTGAA TTTGGTGATG TCAAATGTTT CTAGGGTTTT TTAGTTTGTT 1850

TTTATTGAGA AAATTGATTA TTTATGCTAT AGGTGATATT CTCTTTGAAT 1900

AAACCTATAA TAGGAAATAG CAGACCACAT AAACATCTTT GTAAATATCA 1950

AACCTAATAC ATTTCTTGTC CAGTGATAAA ACAACTGGTA GAATTATTTA 2000

AACACTTTAG ATTTTTAAAT AATAAACATG GCTTTAATTT TTACTGTGTG 2050

TATAGCTACA TGATGAAATT AATTAAATAT TAAGAGGTAA AAAAAAAAAA 2100

AAAAAAAA 2108

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1922 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CGGAAAGCTA CTTCCGGTTG CAGTCATTCT GCCGGGCACC GGCGACCTGT 50

GGCGTGAGCG AGCACAGCCG GAACCCTCCA CTAGCTGGCA TTCCTACCAT 100

CCTTTATAGT GATGCTACAG GACAAAGAGG AATGGATAAA AACATTGGTG 150

AGCAACTCAA TAGAGCATAT GAAGCCTTCC GACAGGCATG CATGGATAGA 200

GATTCAGCAG TAAGAGAGCT ACAGCAAAAG CAGACTGAGA ACTATGAACA 250

AAGAATACGC GAGCAACAGG AACAGCTGTC ATTTCAACAA AACCTAATTG 300

ACAGGCTGAA ATCACAGCTA CTTCTCGTGG ATTCTAGTCG AGATAACAGT 350

TATGGCTATG TACCTTTGCT TGAAGACAGT GACAGAAGGA AGAATAATTT 400

GACCCTTGAT GAACCACATG ATAAAGTGAA ACTAGGAACA CTGAGAGATA 450

AGCAATCAAA GGTGAGACGA CAAGAAGTTT CTTCTGGAAA AGAATCCGCC 500

AAGGGTCTCA ACATCCCTCT GCATCACGAA AGGGATAATA TAGAGAAGAC 550

TTTCTGGGAC CTTAAAGAAG AATTTCATAG GATTTGCTTG CTAGCAAAAG 600

CACAGAAAGA TCACTTAAGC AAACTTAATA TACCAGATAT TGCAACTGAC 650

ACACAGTGTT CTGTGCCTAT ACAGTGTACT GATAAAACAG AGAAACAAGA 700

AGCGCTGTTT AAGCCCCAGG CTAAAGATGA TATAAATAGA GGTATGTCGT 750

GCGTCACAGC TGTCACACCA AGAGGACTGG GCCGGGATGA GGAAGATACC 800

TCTTTTGAAT CACTTTCTAA ATTCAATGTC AAGTTTCCGC CTATGGACAA 850

TGACTCTATT TTTCTACATA GCACTCCAGA GGCCCCGAGC ATCCTTGCTC 900

CTGCCACACC TGAGACAGTG TGCCAGGACC GATTTAATAT GGAAGTCAGA 950

GACAACCCAG GAAACTTTGT TAAAACAGAA GAAACTTTAT TTGAAATTCA 1000

GGGAATTGAC CCCATAACTT CAGCTATACA AAACCTTAAA ACAACTGACA 1050

AAACAAACCC CTCAAATCTT AGAGCGACGT GTTTGCCAGC TGGAGACCAC 1100

AATGTGTTCT ATGTAAATAC GTTCCCACTT CAAGACCCGC CTGACGCACC 1150

TTTTCCCTCA CTGGATTCCC CAGGAAAGGC TGTCCGAGGA CCACAGCAGC 1200

CCTTTTGGAA GCCTTTTCTT AACCAAGACA CTGACTTAGT GGTACCAAGT 1250

GATTCAGACT CAGAGCTCCT TAAACCTCTA GTGTGTGAAT TCTGTCAAGA 1300

GCTTTTCCCA CCATCCATTA CATCCAGAGG GGATTTCCTC CGGCATCTTA 1350

ATACACACTT TAATGGGGAG ACTTAAATCA CGTTTGAAAA CAGACATATC 1400

ATGTTCTCTG TGGTGGTTTT GGATTTGTAA CGCTAGAGAA CGCTTTCTCG 1450

TGAGCCAAAT GTAAGATTGA TTATAAAGTT GTTACTTTAT CTTTTAAGAG 1500

ATCATTTTGT ATAGAACTAT AACTCATTAT ATTATTCATG TTTATACCTA 1550

TAATTTCTAC ATTTCAAAAT TACACATGTG ACTTACAGAG TTATTCAGTC 1600

ATAATTTATG TTTCAAATAG CTAAGTTTAT TGTTTGACTA TTGTGAGATC 1650

TATTAAATTT AGTAATAGCA AATGTTTATA GGATATTCAA ATTTCATTTG 1700

AATTTTTAAT TATTTTTGCT ACAGGTAATA TTCCTTTAAA ATACGTATAT 1750

AACGTACAGA GAATAACAGA CAATATGATC TAAGTAAATG TCGAATCAAT 1800

CATTAGTTGC CCAGGGAAAT TTAAACATTA TAGATCATTT TTAAATAATA 1850

CACATAGTTT TAATTTTTAC TGTGTGTATA GATGCATGAT TAAATGACTT 1900

AAATATTAAA AAAAAAAAAA AA 1922

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2160 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGGCGACCTG TGGCGTGAGC GAGCACAGCC GGAACCCTCC ACTAGCTGGC 50

ATTCCTACCA TCCTTTATAG TGATGCTACA GGACAAAGAG GAATGGATAA 100

AAACATTGGT GAGCAACTCA ATAGAGCATA TGAAGCCTTC CGACAGGCAT 150

GCATGGATAG AGATTCAGCA GTAAGAGAGC TACAGCAAAA GACTGAGAAC 200

TATGAACAAA GAATACGCGA GCAACAGGAA CAGCTGTCAT TTCAACAAAA 250

CCTAATTGAC AGGCTGAAAT CACAGCTACT TCTCGTGGAT TCTAGTCGAG 300

ATAACAGTTA TGGCTATGTA CCTTTGCTTG AAGACAGTGA CAGAAGGAAG 350

AATAATTTGA CCCTTGATGA ACCACATGAT AAAGTGAAAC TAGGAACACT 400

GAGAGATAAG CAATCAAAGG TGAGACGACA AGAAGTTTCT TCTGGAAAAG 450

AATCCGCCAA GGGTCTCAAC ATCCCTCTGC ATCACGAAAG GGATAATATA 500

GAGAAGACTT TCTGGGACCT TAAAGAAGAA TTTCATAGGA TTTGCTTGCT 550

AGCAAAAGCA CAGAAAGATC ACTTAAGCAA ACTTAATATA CCAGATATTG 600

CAACTGACAC ACAGTGTTCT GTGCCTATAC AGTGTACTGA TAAAACAGAG 650

AAACAAGAAG CGCTGTTTAA GCCCCAGGCT AAAGATGATA TAAATAGAGG 700

TATGTCGTGC GTCACAGCTG TCACACCAAG AGGACTGGGC CGGGATGAGG 750

AAGATACCTC TTTTGAATCA CTTTCTAAAT TCAATGTCAA GTTTCCGCCT 800

ATGGACAATG ACTCTATTTT TCTACATAGC ACTCCAGAGG CCCCGAGCAT 850

CCTTGCTCCT GCCACACCTG AGACAGTGTG CCAGGACCGA TTTAATATGG 900

AAGTCAGAGA CAACCCAGGA AACTTTGTTA AAACAGAAGA AACTTTATTT 950

GAAATTCAGG GAATTGACCC CATAACTTCA GCTATACAAA ACCTTAAAAC 1000

AACTGACAAA ACAAACCCCT CAAATCTTAG AGCGACGTGT TTGCCAGCTG 1050

GAGACCACAA TGTGTTCTAT GTAAATACGT TCCCACTTCA AGACCCGCCT 1100

GACGCACCTT TTCCCTCACT GGATTCCCCA GGAAAGGCTG TCCGAGGACC 1150

ACAGCAGGTA ACTGTTTTGC ATTAACAAAT ATTTTATTAT GTGTGAACAC 1200

ACATTTTATC ATACATGTAC AGATACAAAT CTGTTTTAAG TTATCAGGCA 1250

TCCATTTAAA ATTAATGACT ATCCAGAGTT GAGGCTTTCA ATAAAATATG 1300

TAAGTTCTGT ATTCAAGGAC ATGAATTTTG AATGTGACTG CGCTAAAGCT 1350

TCCTTGTGAT ACTGTGGCGT GGCTTTCCCT GCTTCGTCCT CTTCAAGCAC 1400

AGCTTGTTGA CATCAGTGCT CTAATGGATG CTTTATTAAA GTCAGTTACA 1450

GGCAGTAAAT AATTTTTTTA AAACTTGTGT AGGTACACAT AATAATGTGT 1500

AATTTTCCAT AAGTAGATAA TTGCACCAAA TATTCAAAAT AAACTGTCAT 1550

TCAGCCTACT TGTGTTACAT TTCTAGTTAC AGCAGTACAG AGGTCTGTAG 1600

TGTTTGGTTT GTTTACTAAC CTGACACTAA GCAGATATCC TTATACAGTT 1650

TTCAAATAAT CCCTGCACAT GAATACTGTA ATCAAATCTC TTCTTTACTG 1700

TTTGTGAAGC ACAAAGACTT TATAGCCCAT GAATCTAATC CTACCATCCT 1750

TTCTTCCAGA TTCAGGTTCT TTCACAGAAA TATTCCTTTT TGTTAGGAAG 1800

AAAAAAAGTT TTGTTTAATT TCTGAAGGTA AATGCTAAGT GTAGAAATGT 1850

TAAAATAAAT AGAAGCATCT CATTAGAACT TTCAAACATT TGATTTTCTA 1900

TCAGATTAAA AAAAATACTT AATACCTTTG GTTTACGTAT TCCTATCAGT 1950

TATAGGCTTT TTGAACAGCA TGGAAAGAAG CAATAGTGAA GCTGTAGGAT 2000

GTCTTAGTAG TGGGCGTAAG TAGAGATTCT GACAAGTCTT AATTATTAAC 2050

TCTCTTATGT TCCACCCTGT ACCTTATTTC ACTTTATGGT CTCAGCTATA 2100

GTTGCTACCA AATGAAACAA TTAAACAATT TCATGTGTTG CGAAAAAAAA 2150

AAAAAAAAAA 2160